Universidade de Aveiro Departamento de Biologia
Ano 2014

Inês Ferreira Guedes

PATTERNS OF COLONISATION IN AN IMPLANTED MAMMAL CARCASS IN THE DEEP-ATLANTIC OCEAN

PADRÕES DE COLONIZAÇÃO EM CARCAÇAS DE MAMÍFEROS ARTIFICIALMENTE FUNDEADAS NO OCEANO ATLÂNTICO PROFUNDO

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica da Doutora Ana Hilário, Investigadora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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Dedico este trabalho à minha pequena grande família e à enorme família que me permitem escolher...

O júri

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Keywords Atlantic Ocean, colonisation, deep sea, Dorvilleidae, "*Idas*" *simpsoni*, mammal falls

Abstract

Whale carcasses reaching the bottom of the ocean, known as “whale falls”, represent massive organic inputs that provide habitat islands for complex communities of specialised fauna. However, studies about these habitats are mostly restricted to the Pacific Ocean. To investigate the importance of whale falls in the deep-Atlantic Ocean, five mammal carcasses were deployed within the CARCACE project at 1000 m depth in the Setubal canyon (NE Atlantic, west Portuguese margin). In order to describe the fauna associated with the carcasses, investigate the trophic ecology of the metazoan assemblages and analyse the functional morphology of the encountered specialists, bones resulting from the degradation of the carcasses were collected 18 and 28 months after the deployment using a ROV. In this context, the colonisation patterns of two dominant taxa of invertebrates found in the colonising assemblages, the mytilid mussel “*Idas*” *simpsoni* and Dorvilleidae polychaetes were studied. Regarding “*I.*” *simpsoni* the specific goals of this work were to investigate its settlement patterns and to understand its nutritional strategy. The analyses of the populations' size structure showed a continuous settlement and a limitation in growth and adult survival. These limitations are probably due to insufficient energy supplied by the cow bone to maintain chemosynthesis, which is in agreement with the isotopic signatures that indicated a higher contribution of filter feeding than chemosynthesis to their nutrition. Concerning the dorvilleid assemblages, the analyses of species composition disclosed temporal variations associated with distinct food sources at different degradation stages of the bones, as different species presented different isotopic signatures.

Abstract (cont.)

Moreover, species distribution in different microhabitats did not show any relation with the substrate texture, hardness and presence of conspicuous filamentous bacteria on the surface. The morphology of the jaw apparatus of the different dorvilleid species was also analysed in order to investigate the relationship between this structure and trophic ecology. Dorvilleid jaw apparatuses are generally used in taxonomic and phylogenetic studies, but this is the first study on the functional anatomy of these structures. Species with similar jaw characteristics exhibited similar isotopic signatures, suggesting a possible role of the jaw morphology in the specialisation on different resources. However, further studies, using more species from different habitats, are needed to establish this relationship. Overall, these results give significant insights about the ecology of the studied species and into the colonisation patterns of deep-sea mammal carcasses in the Atlantic Ocean.

Palavras-chave carcaça de baleia, colonização, Dorvilleidae, "*Idas*" *simpsoni*, mar profundo, Oceano Atlântico

Resumo

As carcaças de baleia que chegam ao fundo do oceano representam grandes quantidades de matéria orgânica que servem de base a comunidades complexas de fauna especializada. No entanto, a informação disponível sobre este tipo de habitats está praticamente limitada ao oceano Pacífico. Com o intuito de investigar a importância das carcaças de baleia no oceano Atlântico profundo foram fundeadas, no âmbito do projeto CARCACE, cinco carcaças de mamíferos a 1000 m de profundidade no canhão de Setúbal (costa Portuguesa, NE Atlântico). Com o objetivo de descrever a fauna associada às carcaças, investigar a sua ecologia trófica e analisar a morfologia funcional das espécies encontradas, os ossos resultantes da degradação das carcaças foram recuperados utilizando um ROV 18 e 28 meses após o fundeamento. Neste contexto, foi feito o estudo de dois grupos dominantes de invertebrados recolhidos da superfície dos ossos, os mexilhões "*Idas*" *simpsoni* e poliquetas da família Dorvilleidae. Os objetivos específicos deste trabalho relativamente a "*Idas*" *simpsoni*, prenderam-se com o estudo dos padrões de assentamento da espécie e também com a compreensão da sua estratégia nutricional. A análise do tamanho dos indivíduos das populações demonstrou um assentamento contínuo, bem como restrições no crescimento e sobrevivência dos adultos. Estas limitações devem-se, possivelmente, ao facto de os ossos de vaca não disponibilizarem energia suficiente para a realização de quimiossíntese, o que é corroborado pelas análises isotópicas que sugeriram uma maior contribuição da filtração na estratégia nutricional de "*Idas*" *simpsoni*.

Resumo (cont.)

Relativamente à análise da composição das espécies de dorvileídeos esta revelou variações temporais associadas a diferentes fontes de alimentação, o que está de acordo com os resultados da análise dos isótopos estáveis das diferentes espécies, que demonstraram assinaturas isotópicas distintas associadas a cada espécie. Para além disso, a distribuição dos dorvileídeos por diferentes microhabitats não demonstrou qualquer relação com a textura, dureza e a presença de bactérias filamentosas na superfície do substrato. A morfologia do aparelho bocal das diferentes espécies de dorvileídeos foi também analisada com o intuito de investigar a relação entre esta estrutura e ecologia trófica das espécies. O aparelho bocal dos dorvileídeos é, geralmente, utilizado em estudos taxonómicos e filogenéticos, mas o presente estudo constitui a primeira abordagem à anatomia funcional destas estruturas. Espécies com aparelhos bocais semelhantes exibiram assinaturas isotópicas semelhantes, sugerindo uma possível influência da morfologia desta estrutura na especialização de cada espécie em diferentes recursos. No entanto, para estabelecer uma relação direta entre estes dois fatores, será necessário fazer um estudo mais aprofundado, utilizando outras espécies provenientes de outros habitats. No geral, os resultados obtidos neste trabalho, revelam características importantes da ecologia das espécies estudadas, bem como dos padrões de colonização de carcaças de mamíferos no Oceano Atlântico profundo.

"- Agora vou-te contar o tal segredo. É muito simples: só se vê bem com o coração. O essencial é invisível para os olhos...

- O essencial é invisível para os olhos. - repetiu o principezinho para nunca mais se esquecer."

em *O Principezinho*
ANTOINE DE SAINT-EXUPÉRY

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INTRODUCTION

Since the fortuitous encounter with a whale carcass ("whale-fall") in 1987 that deep-sea researchers have documented dozens of sunken whale carcasses, both natural and experimentally implanted (e.g. Smith et al. 1989, Braby et al. 2007, Fujiwara et al. 2007, Lundsten et al. 2010, Amon et al. 2013). Whale-falls deliver large pulses of organic material to the seafloor and serve as habitat islands for unique assemblages of deep-sea fauna that include generalist-scavenging species, chemosynthetic fauna related to those from hydrothermal vents and cold seeps, and bone-specialists (reviewed in Smith and Baco 2003).

Despite the growing interest in these peculiar habitats in the last decade, all long-term studies of deep-sea whale-falls have been restricted to the Pacific. In the Atlantic Ocean, notwithstanding some observations from baited cameras using porpoise and dolphin carcasses (Jones et al. 1998, Kemp et al. 2006) and from shallow water experiments off Scandinavia (Dahlgren et al. 2006, Schander et al. 2010), nothing has been published about the ecology of these habitats. To overcome this gap in the knowledge of whale-falls ecology and their associated fauna, a single parcel of five cow carcasses amounting to 570 kg was deployed at 1000 m depth in the Setubal canyon, off the west central Portugal coast (Hilário et al. (*in press*)). The experimental site was visited and sampled twice with a remotely operated vehicle. Eighteen months after the deployment the totality of the soft tissues had been consumed and the visible remains of the carcasses consisted solely of skeletal material that supported a diverse macrofaunal assemblage, distinct from that of the background, including organic enrichment respondents, bacterial grazers, sulphophiles and bone-specialists (Hilário et al. (*in press*)). Detailed analyses of the second sampling time (28 months) are still underway, but an equally diverse assemblage was observed.

In this thesis I used the material collected from the Setubal canyon, from both sampling times, to investigate the colonisation patters of the mytilid bivalve "*Idas" simpsoni* and dorvilleid polychaetes, dominant members of the faunal assemblages found in the collected bones.

Chapter 1 gives an overview of the underlying theory that the remainder of this thesis will draw upon. It summarizes the history of the exploration of the deep sea and the combination of the environmental settings that characterise it. The ecology of whale-falls is described and the CARCACE project, which aimed at the study of mammal-falls in the deep Atlantic Ocean, is introduced.

Chapter 2 examines the population of the mytilid mussel "*Idas*" *simpsoni* collected from the surface of the retrieved bones. The population size of both sampling times is used to investigate the settlement patterns of this species, and stable isotopic signatures are used to study the nutritional

strategy (filter feeding *versus* chemosymbiosis) used by "*I.*" *simpsoni* in different phases of its development and different stages of bone degradation. The results of this chapter are included in the manuscript "Mammal carcasses attract a swarm of mussels in the deep Atlantic: insights into colonisation and biogeography of an organic-fall chemosymbiotic species" (Annex).

Chapter 3 is focused on the highly diverse assemblages of dorvilleid polychaetes. Temporal and spatial (within the bone) changes in species composition and biomass are analysed, and the nutritional patterns of different species are investigated using stable isotopic analyses. The potential role of jaw anatomy in the specialisation on different resources is also examined; this is the first time that the jaw apparatus is studied from an ecological perspective.

Chapter 4 presents the conclusions of this study by summarising the achievements of the different analyses.

CHAPTER 1. GENERAL BACKGROUND

History of deep-sea biology

Mankind have used and explored the oceans for millennia. However, the exploration of greater depths has a short history because the necessary technologies to investigate it only recently became available. Around 200 years ago, explorers visited the Arctic in a reconnaissance expedition commanded by the British naval officer Sir John Ross that, with a sounding line, found cnidarians and polychaete worms at approximately 2000 m depth (Anderson and Rice 2006). This expedition and its findings are in the basis of history of deep-sea biology (Costello et al. 2010). In 1844, Edward Forbes was one of the first scientists showing interest in understanding species distribution down to the deep-sea. The absence of organisms in Forbes samples deeper than 600 m led to the "Azoic Theory", which postulated that there is little or no life below 600 m (Anderson and Rice 2006).

The "Azoic theory" was in direct opposition to observations of fauna brought up from depths in several regions of the world (Tyler 2003) and stimulated a small group of scientists led by Charles Wyville Thomson to prove the presence of fauna in the deep-sea. Wyville Thomson's work in the Northeast Atlantic established the first ecological observation in the deep sea and directly led to the HMS *Challenger* expedition of 1872 to 1876 (Tyler 2003). This expedition circumnavigated the globe and demonstrated a widespread and varied fauna in the deep sea, as well as taking numerous physical and chemical measurements, setting the foundations for what would be the so called "heroic age" of deep-sea exploration, with expeditions sampling many areas of the world's oceans (Tyler 2003, Ramirez-Llodra et al. 2010). At the end of this period of exploration, in the 1950's, the understanding of the deep sea was one of low biodiversity, no primary production, food-poor, no seasonality and a uniformly cold, dark, calm, quiescent environment.

This view of the deep-sea changed dramatically in the subsequent decades, with increasingly sophisticated sampling methodologies and the ability to collect quantitative samples (Tyler 2003, Ramirez-Llodra et al. 2010). In the 1960's, sampling equipment like the box-corer and the epibenthic sledge allowed quantitative samples of deep-sea communities (Hessler and Sanders 1967). But it was the development of means of observing, exploring and experimenting *in situ* that allowed the discovery of unique habitats, such as hydrothermal vents, cold seeps, whale falls and cold-water corals and a significant increase in our understanding of the biodiversity and functioning of deep-sea ecosystems. In little more than 50 years, the advances in deep-sea technology have led to the development of modern-day submersibles, Remotely Operated Vehicles (ROVs), Autonomous Underwater Vehicles (AUVs) and deep-sea permanent observatories, with ever increasing capabilities for exploration, sampling and experimentation (Danovaro et al. 2014).

Currently, the deep sea is seen as a unique environment, different from all other studied ecosystems, with high species diversity, seasonal input of surface-derived energy for heterotrophic organisms and chemosynthetic primary production at hydrothermal vents, cold seeps and other reduced habitats, such as organic-falls. Yet, only a very limited extent of the deep sea has been explored and many gaps remain in basic knowledge about taxonomy, distribution and ecology of the deep-sea fauna (Costello et al. 2010). Therefore, penetrating and understanding this poorly explored ecosystem is imperative. The deep sea and the specialised adaptations of its organisms have already expanded human's horizons and have led to technological innovation taking to new discoveries that will possibly change long-lasting paradigms.

Environmental setting of the deep-sea

Considered to start at the shelf break, at approximately 200 m depth (Sverdrup et al. 1942), the deep sea is the largest ecosystem on Earth, covering more than 65% of its surface (Tyler 2003). The deep-sea environment is characterised by a combination of abiotic factors (Pradillon and Gaill 2006) that make it so peculiar that is often called "extreme". In summary: pressure is high, temperature is low, there is no light and food input is small.

In the ocean, pressure is a natural parameter forming a continuous gradient from the surface to the ocean floor (Pradillon and Gaill 2006). Pressure increases one atmosphere for each 10 m in the water column, varying, in the deep sea, from 20 atm in the shelf-slope break to more than 1000 atm in its deepest parts. Because pressure can affect organisms both physiologically and biochemically, species living in the deep sea show adaptations that reduce or eliminate the effects of the high pressure on their metabolism, and thus overcome an evolutionary barrier that would otherwise prevent them to successfully inhabit the deep sea (Pradillon and Gaill 2006). Contrarily, salinity is constant and fully marine in the deep sea (approximately 35‰) and, despite some exceptions, this abiotic parameter varies little with time and it appears to be irrelevant to the organisms *in situ* (Thistle 2003).

Temperature generally decreases with increasing depth, reaching approximately 2ºC on the abyssal plain where it is remarkably constant, but the pattern varies with latitude and region (Thistle 2003). Hydrothermal vents are exceptions, with hydrothermal fluids reaching up to 400ºC and the water near them showing variable temperatures (Van Dover 2000). Temperature plays an important role in defining species distributions and diversity in the deep-sea benthos (Young et al. 1997). Low temperatures decrease the chemical reaction rates and therefore, to metabolize at reasonable rates, deep-sea species have biochemical machinery that compensates (Childress 1995). The necessity for

adaptation to low temperatures, like that to high pressure, may constitute a barrier that a warm-, shallow-water lineage must overcome evolutionarily to colonise the cold deep sea.

The oxygen reaches the deep-sea by the descent of surface waters where it enters by exchange with the atmosphere and as a product of photosynthesis in the euphotic zone. The water overlying the deep-sea floor is generally saturated with oxygen, and variations in space and time do constitute a biological challenge for the organisms living in the ocean floor (Thistle 2003). Essentially, the reduction of oxygen concentrations to levels that are problematic for organisms occurs in two situations: 1) when organic material that falls from the euphotic zone is decomposed by aerobic bacteria and is consumed by zooplankton as it sinks, usually between 300 m and 1000 m depth; 2) when the bottom water does not freely exchange with that of the surrounding region, for example, because of a topographic barrier.

One of the first statements about the deep sea was that this was a "calm quiescent" place (Tyler 2003). The water flow at the bottom of the ocean is slower comparing to shallow-water environments (Eckman and Thistle 1991). However, water is never still because of the tidal forces occurring at all depths. This slow movement brings food to all sessile organisms and removes wastes (Thistle 2003). "Benthic storms", or periods of fast flow can also occur and can have important consequences for the fauna, with both positive and negative effects. Such flows prevent the sediments settling from above from covering the entire deep-sea bottom.

Finally, light, or the lack of it, is also a determinant parameter for the biology of the deep-sea floor. The intensity of light decreases exponentially with depth and there is no photosynthetically useful light below 250 m (Thistle 2003). Therefore, the deep sea differs from most of the other ecosystems in that photosynthetic primary production does not occur. Reduced environments, such as hydrothermal vents, cold seeps and wood- and mammal-falls, support primary production based in chemosynthesis, but for the majority of the deep-sea organisms the food must be imported. Apart from precluding photosynthesis, the decrease of light with increasing depth has other consequences for deep-sea species, such as several visual adaptations (e.g. Frank et al. 2012).

Organic-falls in the deep-sea floor

Generally, food is scarce in the deep sea, hence all organic inputs reaching the bottom of the ocean have profound consequences for the ecology of organisms living there. While food characteristics strongly influence the species composition of the community present on the seafloor, the quantity and quality of the food input to the seabed community is the single most important determinant of the abundance of populations in space and time, and may also influence species richness (Gage 2003).

The organic material reaching the bottom of the ocean can be actively or passively transported (Gage 2003). The active transport, also referred as biological transport, is usually ensured by midwater biota that executes vertical migrations between the surface and the deep ocean (Vinogradov and Tseitlin 1983). Zooplankton and nekton, for example, can actively transport organic material through feeding in the surface and defecating at high depths (Longhurst and Harriison 1988, Longhurst et al. 1989). Herein I will focus on the passive sinking of organic material, also known as detrital rain or food falls, that range from small particles to whale carcasses and have different nutritive values as they result from different sources (Gage 2003). Small particles are mostly constituted of dead planktonic organisms, faecal pellets and moults with a reduced size (McCave 1974); the flux of this material is considered the most important source of organic carbon to the deep sea (Gage 2003). Large amounts of plant detritus and macroalgae from terrestrial, shallow-waters or epipelagic origins also reach the deep-sea floor and represent an important food source (Wolff 1976) to which some species became adapted to (Wolff 1979). The different types of organic material reaching the seafloor are illustrated in Figure 1.1.

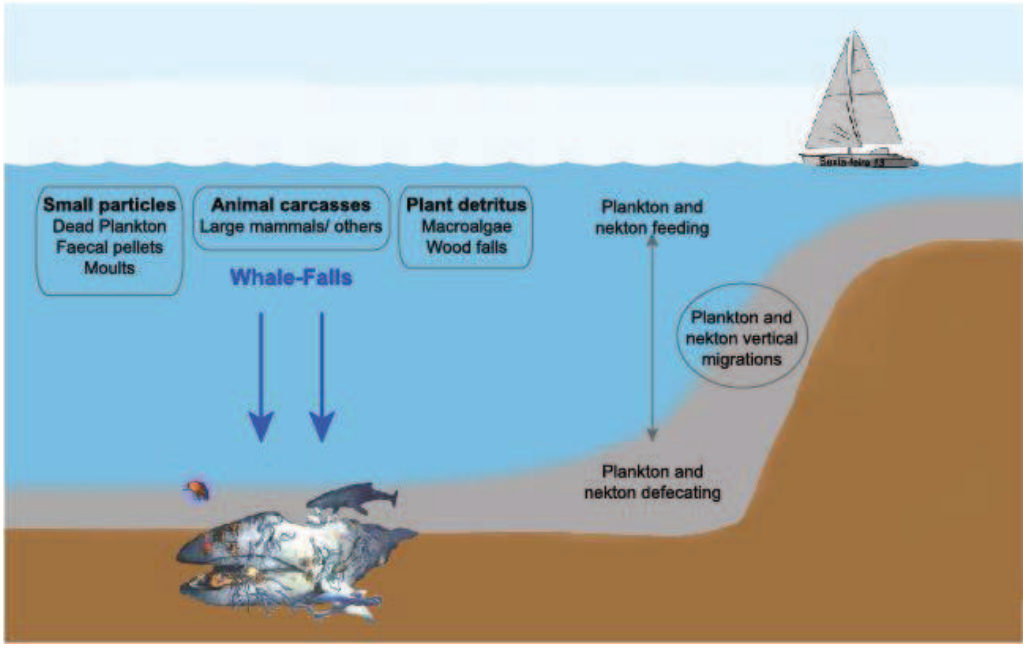

Figure 1.1. Types of food inputs to the ocean floor. Drawing of the whale carcass © Michael Rothman; Image © Jorge Malafaia.

Wood and macroalgae falls have been studied since 1880 as a result of the *Challenger* expedition (Moseley, 1880) and their nutritive value and significance to the carbon cycle in the deep sea was soon recognized (Gage 2003). Since then, *in situ* experiments have become the focus of several studies on the nutritional importance of food-falls of plant origin (Hoyoux et al. 2009) and the response of the deep-sea organisms to their presence (Becker et al. 2009, Bernardino et al. 2010).

These studies suggested that wood and macroalgae falls may provide important environments for the evolution of species frequently associated to organic and sulfide-rich ecosystems in the deep sea (Bernardino et al. 2010) and they can serve as stepping stones for the highly adapted chemosynthetic organisms existing in hydrothermal vents and cold seeps (Bienhold et al. 2013). However, further analyses are needed to clarify several aspects of the global significance of these environments, such as the colonisation and succession patterns around them (Becker et al. 2009).

Large packages of animal origin sinking from the upper water column in the form of carcasses of large animals, such as marine mammals, fish and large invertebrates such as squids, constitute sudden bounties of readily available food attracting an extensive array of specialised organisms (Gage 2003). The overall significance of these highly concentrated organic material has long been debated (Bruun 1957) and the results of early *in situ* experiments using moored seabed cameras focusing on bait, or traps baited with fish or other carcasses, provided convincing evidence of the role of large carcasses as a source of food to deep-sea organisms (Isaacs and Schwartzlose 1975, Thurston 1979, Stockton and DeLaca 1982).

Whale-falls provide the largest amount of biomass from a sinking carcass, and therefore the most persistent food-fall. Smith and Demopulos (2003) estimated that the organic carbon contained in a 40-ton whale is equivalent to that typically sinking from the euphotic zone to a hectare of abyssal seafloor over 100 to 200 years and that the sediments directly underlying a sunken whale carcass (which covers roughly $50m^2$) experience an initial pulse of labile organic material equivalent to approximately 2000 years of background organic carbon flux. Even if the number of large cetacean food-falls to the deep-sea bed may have declined greatly since the development of modern whaling, such food-falls represent a colossal concentration of organic enrichment in a food-poor environment.

The ecology of deep-sea whale falls

The accidental discovery of a skeleton of a 20 m long whale, in 1987, by scientists aboard the submersible Alvin, while mapping the bottom of Santa Catalina Basin (Los Angeles), spurred the interest for whale-falls and their associated faunal communities. The skeleton was half buried in the sediment at 1240 m depth, indicating the death had occurred several years before. One year later the team of scientists returned to the spot for a thorough study. In this second and planned visit, different forms of life were found abundantly distributed in the bones and in the sediment around them - from bacterial mats, and polychaete worms to several types of bivalves and crustaceans. Some of these species were new to science and others had been previously described from the "normal" seafloor or from deep-sea chemosynthetic ecosystems like hydrothermal vents and cold

seeps. The skeleton was then considered an "oasis of life in the vast desert seafloor" (Smith et al. 1989).

The colonisation of a sunken whale carcass begins when it reaches the bottom of the ocean and naturally sunken carcasses are found and often explored. However, it is impossible to predict the death of a whale and therefore impossible to study the successional patterns of these habitats from the beginning. The study of experimentally implanted whale-falls enable scientists to overcome this problem, and in the last two decades it has become common practice to investigate the ecology of these peculiar environments (Smith and Baco 2003, Braby et al. 2007, Fujiwara et al. 2007).

Whale-fall communities undergo successional phases that are characterised by different faunal assemblages. Although such phases may be difficult to distinguish and frequently overlap, time-series studies identified three successional stages of colonisation (Smith and Baco 2003):

1. the "mobile-scavenger" stage begins when the dead animal reaches the ocean floor and it can last up to two years, depending on the carcass size. This phase is characterised by the presence of mobile-scavenger species as sleeper sharks, hagfish, rat-tails and invertebrate scavengers. These organisms remove the soft tissues such as fat, muscles and organs at high rates (40 to 60 $kg.day^{-1}$);

2. the "enrichment-opportunist" stage can also last from months to years. During this phase, the organically enriched sediments and exposed bones are essentially colonised by opportunistic polychaetes and crustaceans that consume remains of the soft tissues. The faunal assemblages at this successional phase exhibit high densities but low species diversity;

3. the "sulphophilic" stage starts after the consuming of all soft tissues and can last for decades. During this stage, anaerobic bacteria breakdown the bone lipids and release sulphide. The sulphophilic stage is characterised by high species richness and trophically complex faunal assemblages that include species that derive their nutrition from chemoautotrophy, via direct grazing or microbial endosymbiosis (Baco and Smith 2003, Bennett et al. 1994, Smith and Baco 2003). The presence of hydrothermal vents and cold seep taxa during this third phase led several authors to suggest that whale-falls may be used by vent and seep animals as "stepping-stones", bridging spatial gaps between ephemeral, sulphide-rich habitats (Smith et al. 1989, Bennett et al. 1994, Naganuma et al. 1996, Smith and Baco 2003, Lorion et al. 2009).

Some studies mention a fourth final stage, the "reef" stage (Jumars and Gallagher 1982, Bennett et al. 1994, Baco and Smith 2003), when, after the depletion of the organic material, the calcified skeleton continues to be explored by suspension feeders that take advantage of flow enhancement and hard substrata (Jumars and Gallagher 1982). This phase may not be reached for many decades, depending on the carcass size (Baco and Smith 2003).

The successional phases described above may exhibit different durations according to the characteristics of the carcasses and the abiotic characteristics of the surrounding environment (Smith and Baco 2003, Braby et al. 2007). For example, the decomposition rates decrease in areas that exhibit low oxygen concentrations, increasing the time of each successional phases (Braby et al. 2007). Therefore, all environmental parameters that characterise the habitat can have an effect on the duration and structure of the different successional phases occurring on these environments (Smith and Baco 2003, Lundsten et al. 2010).

The impact of whaling on deep-sea biodiversity

The history of whaling is very extensive, dating back to prehistoric times (Estes et al. 2006) when human used to hunt whales and other cetaceans to suppress basic needs, using meat for food and oil or blubber for heating. Initially, whaling was performed in small scale but in the last two centuries the increase of whaling activities resulted in a vast reduction in the population sizes of large cetaceans. Whaling has a substantial effect on the rates and geographic distribution of whale carcasses reaching the deep-sea floor (Butman et al.1995). Because whale falls harbour specialised fauna and may serve as dispersed stepping-stones between different chemosynthetic ecosystems in the deep sea, this reduction in whale-falls may have caused species extinctions, and reduced species diversity in deep-sea ecosystems ranging from whale falls to hydrothermal vents (Butman et al. 1995, 1996).

Estimating the biodiversity losses in whale-fall communities, and other deep-sea habitats, caused by intensive whaling is extremely difficult because the data on community structure of these environments is still scarce (Van Dover 2000). However, insights into the effects of fluctuating whale-carcass supply may be gained by studying whale-fall ecology and their biogeography as the global whale populations recover from their hunting-induced lows (Butman et al. 1995).

CARCACE Project

Although studies of whale-falls have intensified greatly in the last decade, the geographical distribution of these studies shows a clear bias towards studies carried out in the Pacific Ocean. The recent discovery of a whale-fall in the deep Southern Ocean extended the knowledge to other ocean basins (Amon et al. 2013), but samples from all major ocean basins are necessary to address questions related to species distribution and biogeography (Smith and Baco 2003).

The CARCACE project (Nationally Funded: FCT - PTDC/MAR/099656/2008) aimed to fill the gap in the knowledge of whale-fall ecology in the Atlantic Ocean, since up to now most

observations came from shallow water experiments off Scandinavia (Dahlgren et al. 2006, Schander et al. 2010). Porpoise and dolphin carcasses have been deployed and studied in the deep Atlantic (Jones et al. 1998, Kemp et al. 2006). However these relatively small food parcels (<100 kg) could not support the successional stages described in the previous section, partially because of their smaller size, partially because the bones of small cetaceans do not have the degree of calcification that is required to ensure slow decomposition and release of bone-lipid reserves (Kemp et al. 2006).

During CARCACE, a single parcel with five cow carcasses amounting to 570 Kg was deployed in the Setubal canyon, off the west central Portugal coast (Figure 1.2, Hilário et al. (*in press*)). The deployment of cow carcasses could generate controversy (Glover et al. 2008, Jones et al. 2008, Vrijenhoek et al. 2008) but still this decision was made because, even though there are numerous records of whale strands on the Portuguese coast, cow carcasses are relatively easy to obtain, allowing to coordinate ship time with the access to mammal carcasses. Further, it was hypothesised, and later confirmed (Hilário et al. (*in press*)), that the high lipid content of cow bones (90%; Lamoureux et al. 2011) in relation to small cetaceans and even whales (up to 10% and 60% respectively; Higgs et al. 2011) can provide enough sulphide for a long enough period of time to reach the sulphophilic stage that can not be sustained by small cetacean skeletons.

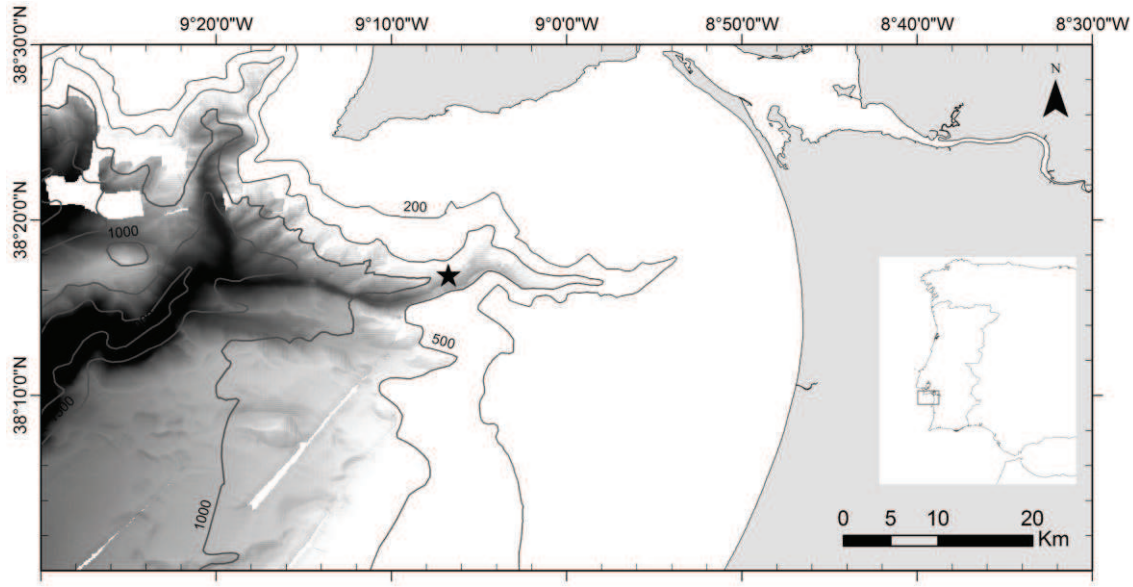

Figure 1.2. Map of the Setubal canyon and location of the experiment site.

The deployment was made using the research vessel NRP *Almirante Gago Coutinho* on 5th March 2011 (Figure 1.3 A), at 1004 m depth, on a migration path of cetaceans. The experimental site was visited on 22nd August 2012 (18 months after the deployment) on board of the same vessel and several exposed bones (Figure 1.3 B) were retrieved with the manipulator arm of the ROV *Luso*

(Figure 1.3 C). On 12th June 2013, 28 months after the deployment of the carcasses, the procedure was repeated on board of the RV *Belgica* and bones were collected with the manipulator arm of the ROV *Genesis*. At both sampling times the bones were brought to the ship in enclosed boxes with natural seawater to maintain the attached fauna (Figure 1.3 D). After the collection, bones were preserved onboard in 96% ethanol or in 4% seawater formalin for DNA sequencing and morphological analyses of the colonising organisms.

Hilário et al. (*in press*) described the colonising community 18 months after the deployment. At this time the totality of the soft tissues had been consumed and the visible remains of the carcasses consisted solely of skeletal material that supported a diverse macrofaunal assemblage, distinct from that of the background. This assemblage included organic enrichment respondents, bacterial grazers, sulphophiles and bone-specialists and its trophic structure showed an overlap of successional stages. At the second sampling time very few bones remained in the site but, although detailed analyses are still underway, an equally diverse assemblage was observed (A. Hilário pers. comm.).

In this thesis I used the material collected from the Setubal canyon, from both sampling times, to investigate the colonisation patters of the chemosymbiotic bivalve "*Idas" simpsoni* and of the opportunistic dorvilleid polychaetes, both dominant members of the faunal assemblages found in the collected bones.

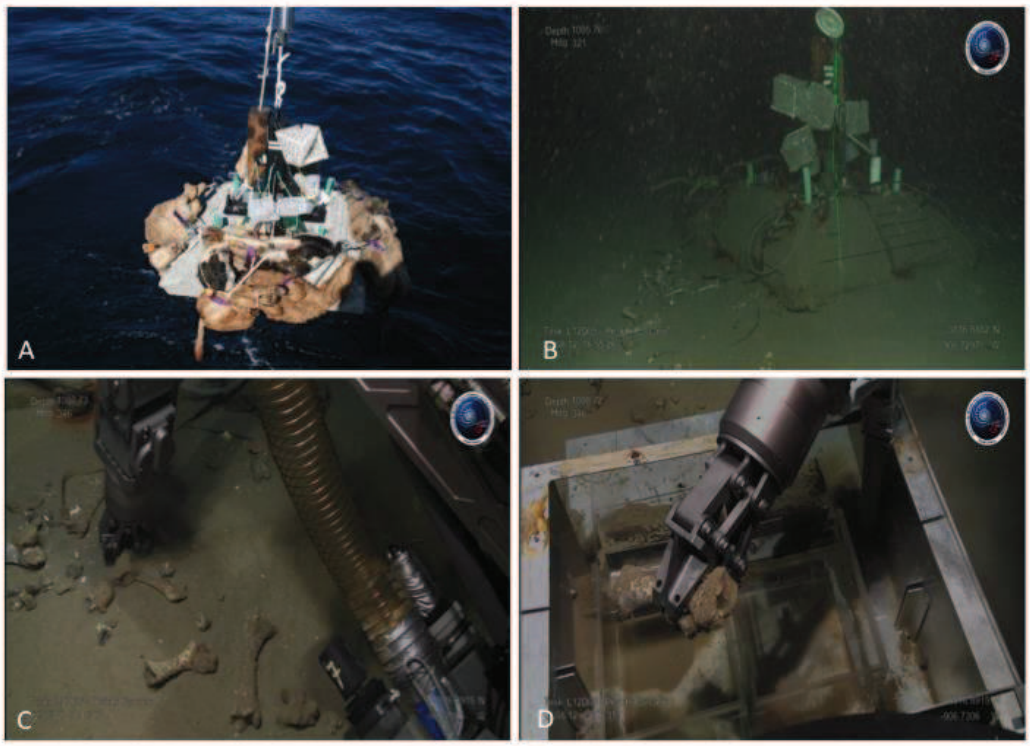

Figure 1.3. A - Deployment of the cow carcasses in Setubal canyon; B - Remaining bones of the deployed carcasses; C – Collection of the bone samples with the ROV *Luso*. D - Manipulator arm of the ROV inserting a bone sample in an enclosed box.

CHAPTER 2. SETTLEMENT AND NUTRITIONAL PATTERNS OF "*Idas*" *simpsoni*

Introduction

Mytilidae is a diverse family of small to large bivalve molluscs within the order Mytiloida. Mytilid mussels have a worldwide distribution and can be found from intertidal to bathyal depths (Buschbaum et al. 2008, Taylor and Glover 2010). In the deep sea, mytilid mussels are ecological important members of chemosynthetic-driven ecosystems and are one of the most well studied groups of macro-invertebrates in these ecosystems (e.g. Dell 1987, Comtet and Desbruyeres 1998, Pond et al. 1998, Eckelbarger and Young 1999, O'Mullan et al. 2001, Won et al. 2003, Becker et al. 2005, Jones et al. 2005, Duperron 2006, Owada 2006, Samadi et al. 2007, Arellano and Young 2011, Lorion et al. 2012, Génio et al. 2012, Thubaut et al. 2013b). Presently, more than 30 species have been described from hydrothermal vents, cold seeps and organic-falls and a large number of unidentified molecular lineages await formal description (Thubaut et al. 2013a).

The ecological success of mytilids at chemosynthetic environments is attributed to their nutritional strategy which involves symbiotic relationships with one or several types of bacteria while maintaining filter-feeding capability, and to long-lived planktotrophic larvae capable of dispersing over large distances (Duperron et al. 2009, Arellano and Young 2009). In addition to these features, their wide distribution and high abundance make mytilid mussels an ideal model-taxon to study the evolution of deep-sea chemosynthetic fauna (Lorion et al. 2010). However, most studies on the ecology of mytilids from the deep sea have been focused on large species that mainly occur at hydrothermal vents and cold seeps, whereas small-sized species associated with organic-falls remain relatively understudied.

Life-history strategies, settlement patterns and feeding modes of small-sized mytilids have been predominantly studied on the Pacific species *Idas washingtonia* (Tyler et al. 2009), *Idas iwaotakii* (Thubaut et al. 2013a), two undescribed *Idas* species (Duperron et al. 2008b), *Adipicola pacifica* (Fujiwara et al. 2010) and *Adipicola longissima* (Duperron et al. 2009). Although 13 mytilid taxa have been reported from organically enriched substrates in the Atlantic, only *Idas modiolaeformis* and *Idas argenteus,* both inhabiting wood remains, have been investigated (Dean 1993, Duperron et al. 2008a, Ockelmann and Dinesen 2011, Gaudron et al. 2012), highlighting the lack of studies on this group of mussels associated with mammal-falls in the Atlantic Ocean.

A high abundance of "*Idas*" *simpsoni* (Figure 2.1) was found on the bones collected 18 and 28 months after the deployment of the mammal carcasses in the Setubal canyon, providing an excellent opportunity to study several aspects of the ecology of small mussels from this habitat in

the deep-Atlantic Ocean. "*Idas*" *simpsoni* belongs to a group of small mussels frequently associated to wood and bone substrates that have been provisionally ascribed to the new genus *Nypamodiolus* (Thubaut et al. 2013a). However, since this classification has not been formally accepted, the mytilid mussels found in the collected bones are referred as *"Idas" simpsoni* under inverted commas, as suggested by Génio et al. (*in press*).

Herein I analyse the population size-structure of "*Idas*" *simpsoni* from both sampling times in order to understand settlement patterns of this species and I use stable isotope analyses to investigate possible shifts in the nutritional strategy of "*I.*" *simpsoni* (chemosymbiosis *versus* filter feeding) between development stages and sampling times.

Figure 2.1. Clump of live mussels settled on a cow bone from the experimental mammal fall at Setubal canyon, NE Atlantic. Scale bar: 1 mm. Génio et al. (*in press*).

Material and Methods

Bone imaging and sorting

All specimens of *"Idas" simpsoni* were retrieved from the surface of four bones resulting from the CARCACE experiment. Two bones (B and C) were collected 18 months after the deployment and the other two (G and H) 28 months after the deployment. After the removal of all mussels the bones were imaged using a computerized tomography scanner (General Electric BrightSpeed 16). Image acquisition was performed in helicoid mode with thicknesses cut of 0.625 mm, reconstruction interval of 0.310 mm, 100 kVp, 100 mAs, Pitch of 0.562:1, and reconstruction algorithms Standard and Bone. The bone-reconstructed images (Figure 2.2) were used to estimate the area of water-exposed surface and this area was used to calculate the density of individuals (individuals.cm^{-2}) in each bone sample.

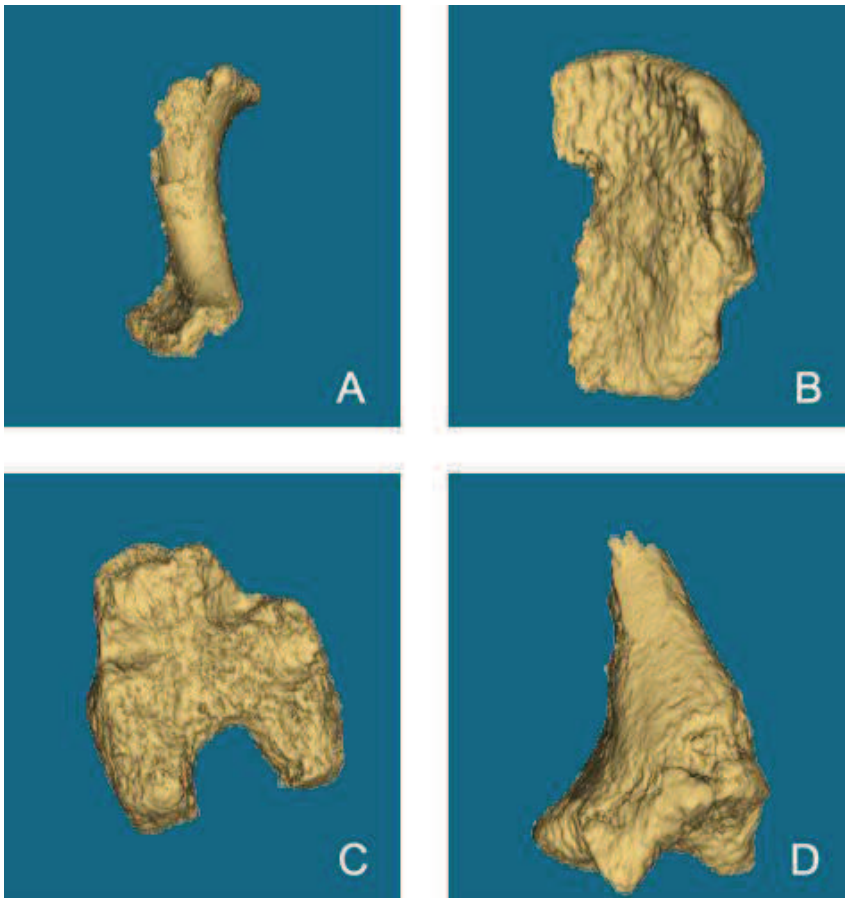

Figure 2.2. 3D images of the bones acquired using a computerized tomography scanner. A – Bone B, 18 months; B – Bone C, 18 months; C – Bone G, 28 months; D – Bone H , 28 months.

Population size structure

The shell length of each mussel was measured under a stereomicroscope (Leica S8APO) fitted with an ocular micrometer (Figure 2.3). Length-frequency distributions were plotted using a 200µm length-class interval, chosen according to three criteria: 1) most size classes must have at least five individuals, 2) the number of adjacent empty classes must be minimized, and 3) the length-class interval should be larger than the error on the length measurements (12.5 µm, 31.24 µm and 50 µm, respectively at 40x, 16x and 10x amplifications) (Kelly and Metaxas 2008). Length-frequency distributions were compared to a normal distribution using a Kolmogorov-Smirnov one-sample test. Within a deployment period, length-frequency distributions were compared between pairs of bone samples using Kolmogorov-Smirnov two-sample tests, and the same test was used to compare distributions between the two sampling phases (pooled samples from 18 and 28 month deployments). Modal decomposition of length-frequency distributions was done using the MIXDIST package (v. 0.5-4) developed for the R environment (v. 3.0.0) (MacDonald and Du 2008), assuming that mytilid sizes follow a Gamma distribution within a single cohort. MIXDIST

uses maximum likelihood criteria to provide the best mathematical fit between theoretical and observed mixtures of frequency distributions.

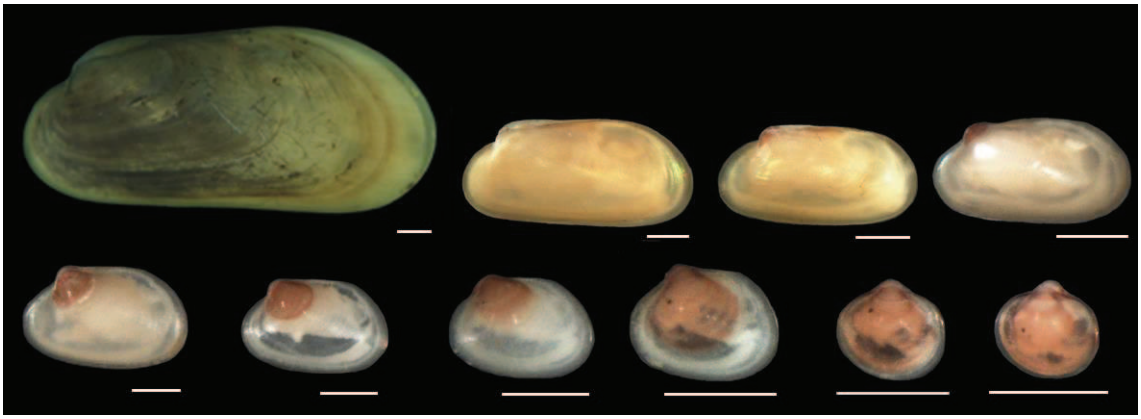

Figure 2.3. Digital photographs of "*Idas*" *simpsoni* collected from the surface of cow bones at Setubal canyon, NE Atlantic. Plantigrade stage is shown in the bottom right corner.
Scale bars: upper row - 1mm; lower row – 500 µm. Génio et al. (*in press*).

Isotopic composition

Analyses of the isotopic composition of the soft tissue of "*I.*" *simpsoni* were performed on specimens from different development stages and from different sampling times. Developing males were recognized in individuals as small as 1.8 mm, and the largest individual found in the whole experiment (13.8 mm shell length) showed female reproductive features (A. Hilário, personal observation). Considering these features, specimens were separated into post-larvae (individuals with a shell length up to 1.8 mm) and adults (individuals with a shell longer than 1.8 mm). The samples were oven-dried and homogenized using a mortar and pestle and acidified by fumigation (Harris et al. 2001): the samples and a beaker with 100 ml of concentrated HCl (12M) were placed in a desiccator for 48 hours at 60ºC. Nitrogen and carbon stable isotope analyses were carried out in MARINNOVA, CIIMAR (Porto, Portugal) using the EA-IRMS method (elemental analysis – isotope ratio mass spectrometry) performed with an Organic Elemental Analyser (*Flash 2000*) coupled to a mass spectrometer *Delta V Advantage* (*Thermo Scientific* via *Conflo IV*). The C/N atomic ratios were calculated from the percentages of organic carbon and nitrogen. The isotopic composition of the samples was expressed by the relative difference between isotopic ratios in the sample and that rations in the conventional standards (atmospheric N_2 and Peedee belemnite marine limestone (PDB):

$\delta^{15}N$ or $\delta^{13}C(‰) = [(Rsample/Rstandard) -1] \times 1000$

where $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$.

All graphs and statistical analyses carried out in this study were performed with GraphPad Prism (v. 6.0).

Results

Mussel density and size structure

The estimated values of the water-exposed surface area of each bone sample acquired using computerized tomography scanner images are presented in Table 2.1. Bone C presented the smaller surface having 259.47 cm^2 of exposed surface and bone H the larger with 537.39 cm^2 of bone surface area.

A total of 4895 individuals were sorted from the four bones. Bone B was the sample with the lower number of individuals (N=883), followed by bone G (N=995), bone H (N=1389) and bone C (N=1628). Mussel density (individuals.cm^{-2}) ranged from 1.92 in bone B to 6.27 in bone C as presented in Table 2.1. Within the samples collected 18 months after the deployment there was a high discrepancy between mussel densities found in the two bones (1.92 and 6.27 ind.cm^{-2}), whereas samples from 28 months revealed similar densities (2.05 and 2.58 ind.cm^{-2}).

The shell length of all mussels ranged from 425 to 6375 µm and from 375 to 7000 µm in the bones collected 18 and 28 months after the deployment, respectively (Table 2.1 and Figure 2.4).

Table 2.1. Information used to obtain mussel's density in each bone sample.

	Bone surface area (cm^2)	Total number of individuals	Number of plantigrades	Mussel density (individuals.cm^{-2})	Shell length range (µm)
Bone B	459.06	883	8	1.92	450 - 2550
Bone C	259.47	1628	12	6.27	425 - 6375
18 months	--	2511	20	--	425 - 6375
Bone G	485.34	995	46	2.05	400 - 4900
Bone H	537.39	1389	267	2.58	375 - 7000
28 months	--	2384	313	--	375 - 7000

The results of Kolmogorov-Smirnov one sample test showed that all length-frequency distributions were significantly different from the normal distribution ($p<0.0001$). The length-frequency distributions in bones collected 18 months after the deployment were significantly different (Kolmogorov-Smirnov two sample test, $p<0.0001$) but both showed an unimodal structure characterised by a large number of individuals with approximately 1 mm length. A total of 20 plantigrades (recently settled individuals) were collected from the 18 months samples, 8 from bone B and 12 from bone C, representing less than 1% of the mussels from this sampling time (Table 2.1). The length-frequency distributions from the 28 months samples were also significantly different (Kolmogorov-Smirnov two sample test, $p<0.0001$) but, in contrast to the 18 months samples, exhibited a bimodal structure. A total of 313 plantigrades were collected, 46 from bone G and 267 from bone H, representing 13.1% of the total number of mussels from 28 months samples (Table 2.1). A very high proportion (82%) of mussels in bone G were large specimens of about 1.6 mm length, while bone H had nearly 50% of small mussels with less than 600 μm.

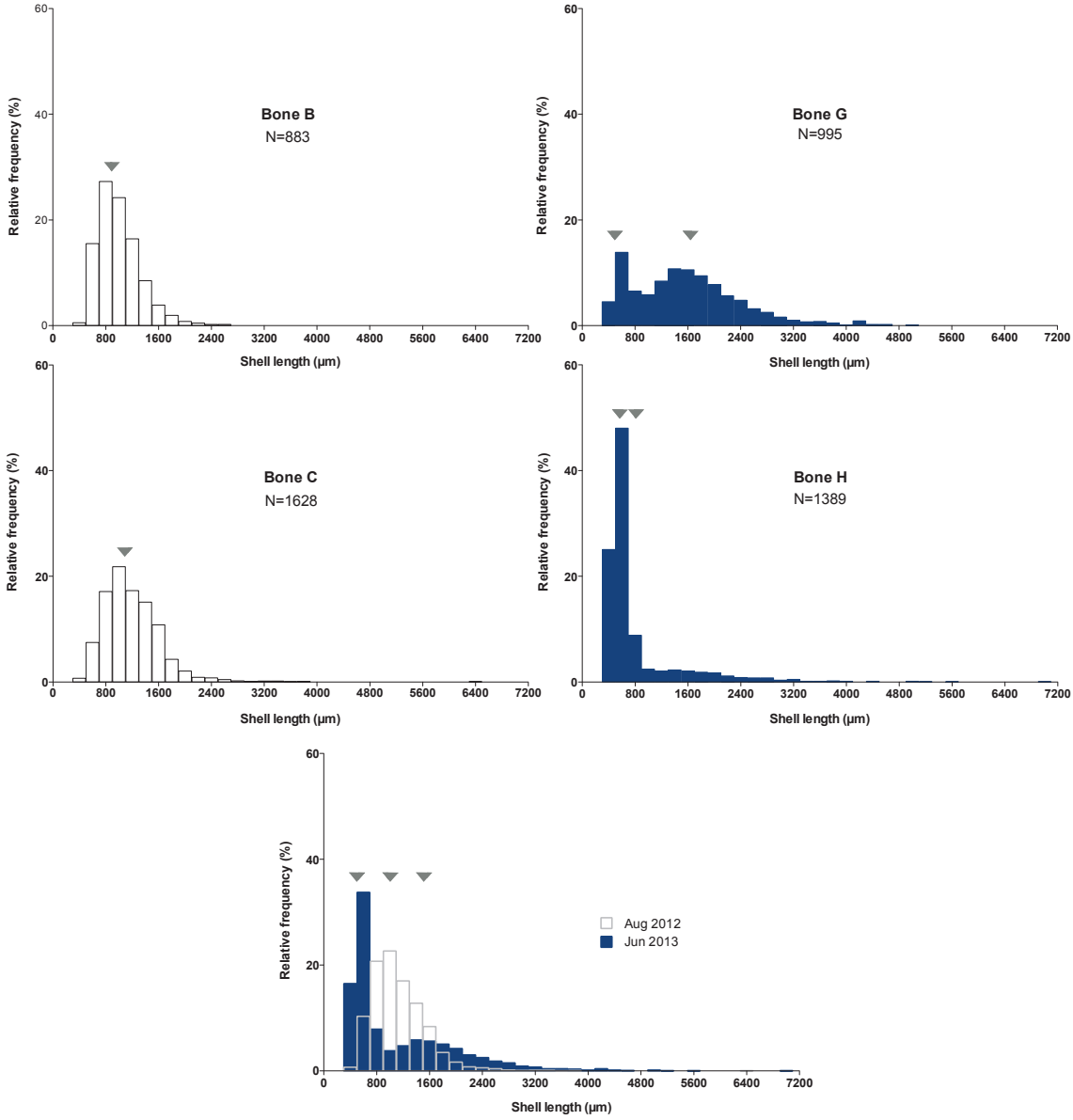

Figure 2.4. Length-frequency distribution of "*I.*" *simpsoni* collected from the surface of cow bones at Setubal canyon, 18 (white) and 28 (blue) months after deployment (respectively, bones B and C in Aug 2012, and bones G and H in Jun 2013). Grey arrows indicate the mean length of modal components identified with MIXDIST package; N is the total number of individuals in the distribution. Génio et al. 2014.

Isotopic signatures

The stable isotopic analyses performed on post-larvae and adults sampled 18 and 28 months after the deployment exhibited $\delta^{15}N$ values ranging from 3.6‰ to 5.0‰ and $\delta^{13}C$ ranging from -19.6‰ to -10.3‰ (Table 2.2 and Figure 2.5).

"Idas" simpsoni post-larvae displayed higher absolute mean values of ^{15}N and ^{13}C than adults (Figure 2.5). Post-larvae exhibited a nitrogen signature of 4.8‰ (Stand. deviation = 0.2) and a carbon signature of -15.5‰ (Stand. Deviation = 3.9); adults showed a nitrogen signature of 4.1‰ (Stand. Deviation = 0.4) and a carbon signature of -10.9‰ (Stand. Deviation = 1.4).

Mussels collected 18 months after the deployment showed mean nitrogen and carbon signatures of 4.5‰ (Stand. Deviation = 0.1) and -12.2‰ (Stand. Deviation=1.0), respectively. Mussels collected 28 months after the deployment showed a mean nitrogen signature of 4.3‰ (Stand. Deviation = 0.8) and a mean carbon signature of -15.2‰ (Stand. Deviation = 4.4). The stable isotopic composition of bone C (18 months) and bone H (28 months) was also analysed, showing a mean nitrogen signature of 6.3‰ (Stand. Deviation = 0.7) and a mean carbon signature of -21.9‰ (Stand. Deviation = 0.3) (Figure 2.5).

In Figure 2.6 a summary is presented of the results obtained in this study in relation to isotopic signatures of the genus *Idas* found in previous studies.

Table 2.2. Stable isotope signatures for mussels in different development stages collected 18 and 28 months after the deployment. PL – Post larvae; A – Adults

		$\delta^{15}N$	$\delta^{13}C$
18 months	PL	4.6	-11.7
		4.6	-12.8
	A	4.4	-13.2
		4.5	-11.2
28 months	PL	5.0	-19.6
		5.0	-18.1
	A	3.6	-10.3
		3.7	-12.9

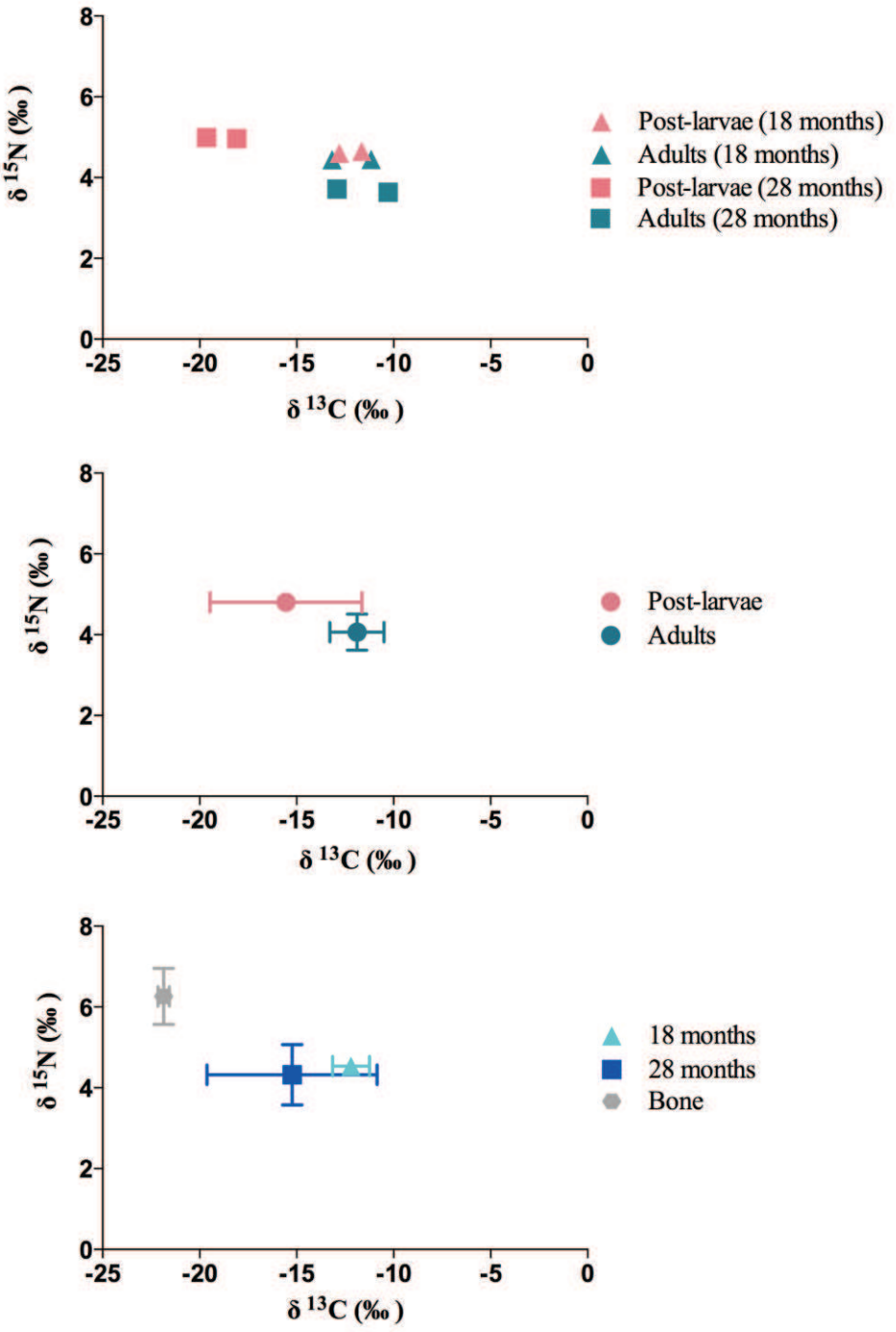

Figure 2.5. Stable isotope signatures for post-larvae and adult mussels collected 18 and 28 months after the deployment (top); mean and standard deviation of stable isotope values in "*Idas*" *simpsoni* post-larvae and adults (middle); mean and standard deviation of stable isotope values of "*Idas*" *simpsoni* collected 18 and 28 months after the deployment (bottom); "Bone" represents the isotopic composition (mean and standard deviation) of bone C and H.

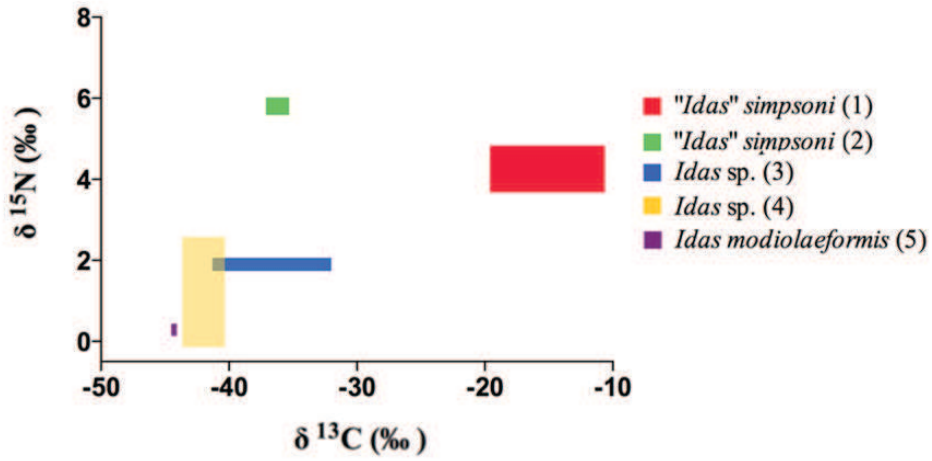

Figure 2.6. Isotopic $\delta^{15}N$ and $\delta^{13}C$ values from the literature for the genus *Idas*. 1 – Mammal carcass; This study. 2 - Cold- seep; Ritt et al. 2012; Phylogenetic analyses performed by Génio et al. 2014 identified *Idas* sp. from the Marmara Sea as *Idas simpsoni*. 3 - Napoli Mud Vulcano; Carlier et al. 2010. 4 - Amsterdam Mud Vulcano; Carlier et al. 2010. 5 - Cold seep; Olu-Le Roy et al. 2004.

Discussion

Settlement patterns

Bone C, collected 18 months after the deployment, showed a much higher density of "*Idas*" *simpsoni* than the three other analysed bones, which may result from differences in the chemical composition of the bone or from ecological interactions, such as inter-specific competition. The chemical composition of the different bones was not analysed and therefore it was not possible to establish a relation between higher densities of "*I.*" *simpsoni* and bone chemical composition. Regarding ecological interactions, the whole invertebrate community of each bone was not investigated, but it is worth noting that bone C presented the lower density of dorvilleid polychaetes (Chapter 3).

The size-frequency distribution of individuals collected 18 months after the deployment exhibited an unimodal pattern suggesting a continuous settlement of new individuals into the population (Kelly and Metaxas 2008). In contrast, the distribution of the length-frequency of the 28 months samples revealed distinct peaks, indicating a discontinuous settlement pattern. However, for both sampling times the maximum shell length was similar (~7 mm). Different bones, irrespective of the sampling time, showed dominance of mussels of distinct size-classes, which suggests spatial segregation of sizes. Recently settled individuals (plantigrade stage) occurred at both sampling times, yet they occurred in much higher numbers in the 28 months samples: 13% of the total individuals from bones G and H, compared to 1% in bones B and C. Using the shell growth (766-869 $\mu m.30d^{-1}$) estimated by Génio et al. (*in press*) settlement events probably happened in March 2011, December 2011 to January 2012, August to October 2012, and June 2013.

Although seasonal reproduction has been reported for large mussels from hydrothermal vents and cold seeps (Tyler et al. 2006, Dixon et al. 2006), studies on reproduction of small mussels inhabiting organic falls could not confirm seasonality due to the lack of samples covering different seasons (Tyler et al. 2009, Gaudron et al. 2012). The possibility of continuous recruitment of "*I.*" *simpsoni* was suggested in carbonate crusts in the Marmara Sea (Ritt et al. 2012). Regardless of the spawning periodicity, viable larval pools may be kept for more than a year due to the long planktonic larval duration estimated for deep-sea mytilids [1-5 months in *I. modiolaeformis* (Gaudron et al. 2012) and up to 13 months in "*Bathymodiolus" childressi* (Arellano and Young 2009)], allowing a continuous settlement if suitable habitat is available.

Intraspecific competition for space and food, especially between adults and larvae, including larviphagy, plays an essential role on the habitat availability for successful larval settlement (Comtet and Desbruyeres 1998). As large individuals become less numerous, the amount of suitable habitat increases leading to the establishment of another generation of a numerically dominant size-class (Dean 1993). *"Idas" simpsoni* is known to attain shell lengths up to 45 mm (Warén and Carroza 1990). However, in this study only a reduced number of individuals showed lengths higher than 3 mm, which indicates that growth and survival is limited. This restraint in growth and survival, possibly caused by insufficient energy and limited space provided by cow bones in comparison with other habitats (e.g. carbonate crusts) is likely to explain the observed differences of size-class abundances in each bone.

Trophic ecology

The stable isotopic signatures of ^{15}N and ^{13}C are powerful tools to perform studies on trophic ecology of marine environments (Sulzman 2007). The $\delta^{15}N$ values are characteristic of different trophic levels wherein producers and first consumers are depleted in ^{15}N. $\delta^{15}N$ tend to increase with trophic level (3 to 4‰ at each position in the food web) and moreover the nitrogen signatures of deep-sea invertebrates range from -2 to 10‰. Organisms exhibit a $\delta^{13}C$ according to their nutritional sources and these values are indicatives of the origin of the organic carbon. $\delta^{13}C$ of -22‰ to -15‰ indicate planktotrophic originated carbon whilst values < -22‰ suggest a chemosynthetic pathway (Van Dover 2007, Carlier et al. 2010).

The isotopic analyses of "*Idas*" *simpsoni* collected within this study evinced that these organisms derive their nutrition from a chemosynthetic source, at both sampling times and in different development stages. The obtained values for $\delta^{15}N$ were positive for all samples, ranging from 3.6 to 4.9‰ possibly reflecting the absence of symbiosis with chemoautotrophic bacteria in both post-larvae and adults from the two sampling times (Van Dover 2007). Regarding the $\delta^{13}C$, the obtained values (-19.6 to -10.3‰) were slightly higher than those from previous studies on the trophic

ecology of deep-sea chemosymbiotic mussels in which the carbon composition varies from -44.6‰ to -21.3‰ (Fisher 1990, Pond et al. 1998, Trask and Van Dover 1999, McKiness et al. 2005, Ritt et al. 2012, Carlier et al. 2010, Olu-Le Roy et al. 2004). Particularly for the genus *Idas* previous $\delta^{13}C$ values range from -44.6 to –32.0‰. The disparity between these values and those encountered in this study may result from a reduced contribution of symbiosis *versus* filter-feeding in the nutrition of "*I.*" *simpsoni* herein analysed. Although Génio et al. (*in press*) found sulphur-oxidizing bacterial symbionts in "*I.*" *simpsoni* from the samples used in this study, the amount of bacterial detected in the mussels' gill was minimal (C. F. Rodrigues, personal observation).

The comparison of the isotopic composition of different development stages showed that post-larvae have higher values of $\delta^{15}N$ than adults. It has been suggested that deep-sea mytilids have a planktotrophic larval stage that may spend more than one year in the surface waters (Arellano and Young 2009, Arellano et al. 2014, Gaudron et al. 2012). Therefore, the slightly higher values of $\delta^{15}N$ found in the post-larvae in comparison with adults may be due to planktotrophy of the larval stage of "*I.*" *simpsoni* while in the water column. However, the mean value of $\delta^{13}C$ found in post-larvae was more negative than in adults, which is inconsistent with the argument presented for the differences found in the values of $\delta^{15}N$. Adults collected at both sampling times and post-larvae from the 18 months samples had similar $\delta^{13}C$ values (-13.2‰ to -10.3‰) but post-larvae collected 28 months after the deployment had relatively lower values (-19.6 to -18.1‰) resulting in the more negative mean value of $\delta^{13}C$ in post-larvae. Although post-larvae were defined as individuals with shell-length lower than 1.8 mm, the samples from 28 months had a much higher proportion of plantigrades, which have a thinner shell that may be more rapidly eliminated by fumigation. The thicker shell of adults and larger post-larvae may not have been totally eliminated by this acidification method leading to more positive values. Between the two sampling times the mean isotopic signatures were similar and the large standard deviation found in $\delta^{13}C$ values from the 28 months samples was probably due to the acidification method as already explained.

Conclusion

The samples collected from the experimental deployment of mammal carcasses in the Setubal canyon (NE Atlantic) disclosed surprisingly high abundances of the small chemosymbiotic mussel "*Idas*" *simpsoni*. Analyses of the population size structure suggested continuous settlement and limitation in growth and adult survival that may be due to limited space and insufficient energy provided by the cow bone in comparison with other reducing habitats. Deep-sea chemosymbiotic mussels host chemoautotrophic bacteria while maintaining their filter-feeding capacity. Although sulphide-oxidizing symbionts have been previously found in *"I." simpsoni* sampled from the bones

herein analysed, stable isotopic signatures indicated a higher contribution of filter-feeding to their nutrition. The energetic gain from filter-feeding however, may not be enough to allow growth to the maximum sizes reported for this species.

Adults and post-larvae of *"I." simpsoni* presented different isotopic signatures that suggest different nutritional sources related to larval migration to surface waters. However, these results must be interpreted cautiously because of possible methodological constraints related to the small size of analysed individuals.

CHAPTER 3. SPECIES COMPOSITION, DISTRIBUTION AND NUTRITIONAL PATTERNS OF THE DORVILLEIDAE ASSEMBLAGES

Introduction

Dorvilleidae Chamberlin, 1919 is a diverse polychaete family within the order Eunicida, comprising at least 38 genera (Read 2014a). These organisms exhibit a rounded prostomium usually with a simple or articulated pair of dorsolateral antenna and a pair of lateral palps or, rarely, these structures may be absent (Jumars 1974). Dorvilleids are benthic worms distributed from the tropics to the poles that can be found in marine and estuarine ecosystems in a wide range of depths (Hilbig and Blake 1991). With 62 formally described species, *Ophryotrocha* Claparède & Meczinikow, 1869 is the most speciose genus within this family (Read, 2014b).

Ophryothrocha are opportunistic organisms that are frequently dominant in polluted and eutrophic sediments (Dahlgren et al. 2001, Brooks et al. 2003, Hall-Spencer et al. 2006), but also in deep-sea reduced habitats as hydrothermal vents (Blake 1985, Van Dover et al. 1988, Mullineaux et al. 2003, Paxton and Morineaux 2009), hydrocarbon seeps (Weiss and Hilbig 1992, Sahling et al. 2002, Robinson et al. 2004, Levin et al. 2012) and whale-falls (Smith et al. 1998, Dahlgren et al. 2006, Fujiwara et al. 2007, Wiklund et al. 2009b, 2012, Amon et al. 2013). *Ophryotrocha* have a short life cycle (Sella and Ramella 1999) and are relatively easy to maintain in laboratory cultures (Åkesson 1967, 1973) and are therefore frequently used as a model organism in comparative and evolutionary biology of marine invertebrates (reviewed by Thornhill et al. 2009). Although this is a relatively well-studied genus, recent exploirations of the deep sea have led to the discovery of several new species (Wiklund et al. 2009a, 2012, Taboada et al. 2013) and raised new question about their evolution and ecology (Thornhill et al. 2012, Levin et al. 2013).

Hilário et al. (*in press*) reported the first account on the biodiversity of the mammal carcasses implanted in the Setubal canyon referring exclusively to bones collected 18 months after the deployment. Six dorvilleid species belonging to the genera *Ophryotrocha* (5 species) and *Parougia* (1 species) were reported, but subsequent analyses of bones collected 18 and 28 months after the deployment revealed one further species of *Ophryotrocha* (A. Ravara, personal observation). All seven dorvilleid species are new to science except *Ophryotrocha scutellus* that has been described from shallow-water habitats, including a whale-fall, off Scandinavia (Wiklund et al. 2009a). The co-occurrence of more than one species of dorvilleids in reduced environments is not completely unexpected. Levin et al. (2003) reported the presence of six dorvilleid species living in different substrates in methane seeps in California and Oregon coast, and five species have been found

associated to whale bones and reduced sediment at an implanted whale carcass in the shallow North Atlantic (Wiklund et al. 2009a). However, the presence of seven species in the exact same substrate (bone) at the same time is remarkable.

It has been suggested that dorvilleid species co-occur by niche partitioning through trophic patterns and habitat preference/tolerance (Levin et al. 2003, 2013). Niche partitioning, although at a microhabitat scale may also explain the high dorvilleid diversity found in the Setubal experiment as bones presented several distinctive patches with different textures and covered by different microbial communities (Figure 3.1). In order to test this hypothesis and to understand what drives dorvilleid diversity in the bones from the mammal carcasses implanted in the Setubal canyon I analyse the following aspects of the dorvilleid assemblage:

1. species density, composition and biomass at 18 and 28 months after the carcass deployment;
2. species composition in different microhabitats within the bones;
3. stable isotope signature of different species.

Moreover, I investigate the importance of jaw morphology in the possible specialisation of dorvilleid species on different resources. Dorvilleidae are characterised by having a compound jaw apparatus consisting of ventral mandibles and dorsal maxillae with complex hard elements that are formed as sclerotized projections of the pharyngeal cuticle (Purschke 1987). These structures have been accepted as one of the most important characters for species identification (Purschke 1987) and have undergone numerous investigations, but their role in the trophic ecology of the different species has been overlooked.

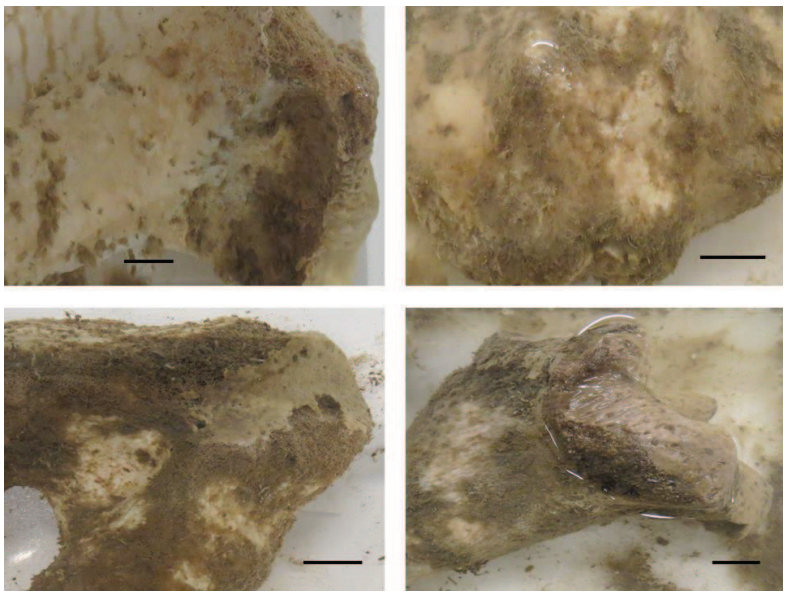

Figure 3.1. Detail of different surfaces of the bone samples showing different patches colonised by *Ophryotrocha* sp. Scale bars: 1cm.

Material and methods

Identification of bone microhabitats

The four bones were carefully analysed under a stereomicroscope and each of them was divided in different areas according to the hardness and texture of the surface and the presence of conspicuous filamentous bacteria. A total of six areas were identified:

1. "Featureless and hard" (FeatH): slightly degraded periosteum, smooth/uniform, hard and brown surface;
2. "Rugged and soft" (RS): moderately degraded osteum, rugged, soft and white surface;
3. "Reticulate and friable" (RetF): highly degraded osteum, reticulate, soft and dark brown surface;
4. "Featureless and friable" (FeatF): articulate surfaces of the bone, smooth/uniform, soft and grey (darker than RS and lighter than the other areas) surface;
5. "Reticulate and hard" (RetH): spongy bone, reticulate, hard and brown (lighter than FeatH) surface;
6. "Filamentous bacteria" (BF): fluffy bacterial mat with sediment; usually dark brown.

Areas with conspicuous filamentous bacteria (BF) were only found in bones collected 28 months after the deployment.

Dorvilleidae species density, composition and biomass

All specimens were carefully removed from the surface and below the surface of the different areas of the bone to a maximum of 5 mm. Several dissection tools (e.g. tweezers, forceps, scalpels) were used depending on the hardness of the surface. After the collection, all the individuals were counted and grouped in species according to their morphology, including the jaw apparatus, palps and antennas (Figure 3.2). Further, species that had different jaw apparatus between their development stages were separated in adults and juveniles.

The density (number of individuals.cm^{-2}) was calculated for each bone sample using the water-exposed surface area as described in Chapter 2.

To compare the species composition between different microhabitats multidimensional scaling analyses were performed for each sampling time using the software PRIMER (v. 6.1.11). The abundance data were normalized using a fourth root transformation and the similarity was calculated using Bray-Curtis measure.

For estimating the mean individual weight of each species collected from each of the six putative microhabitats I used an indirect method adapted from (Heip et al. 1985) in which the following formula was used:

$B = (LxW^2)/(1.6x10^6)$

where *B* is the biomass (µg) per individual, *L* is the polychaete total length (µm) and *W* is the maximum body width. To obtain these parameters, pictures of all "undamaged" specimens were taken under a stereomicroscope using the Leica Acquire image software associated to the camera mounted on the microscope. The software ImageJ was used to measure the total length and the maximum width. The mean individual biomass was then used to calculate the total biomass of each species in each area.

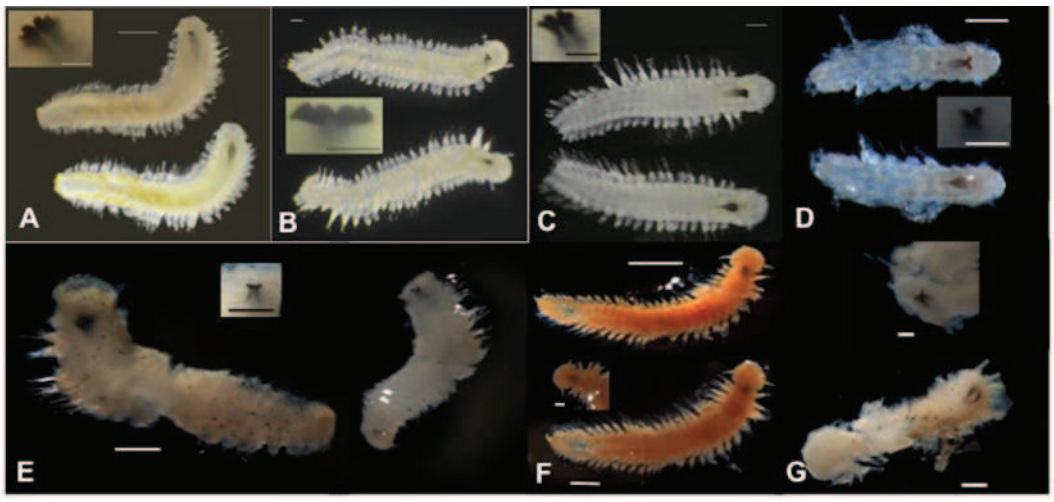

Figure 3.2. Morphological details of Dorvilleidae species. A: *Ophryotrocha* sp. 1 (scale bar - whole animal: 0.5mm, mandible: 0.05mm); B: *Ophryotrocha* sp. 2 (scale bar - whole animal: 0.1mm, mandible: 0.05mm); C: *Ophryotrocha* sp. 3 (scale bar - whole animal: 0.1mm, mandible: 0.05mm); D: *Ophryotrocha* sp. 4 (scale bar - whole animal: 0.1mm, mandible: 0.1mm); E: *Ophryotrocha* sp. 5 (scale bar - whole animal: 0.2mm, mandible: 0.2mm); F: *O. scutellus* (scale bar - whole animal top: 1mm, whole animal bottom: 0.5mm, mandible: 0.2mm); G: *Parougia* sp. (scale bar - whole animal: 0.2mm, mandible: 0.1mm).

Isotopic composition

Due to the small size of the dorvilleid species found in this study analyses of the isotopic composition were only possible by pooling specimens of the same species from different bones and putative microhabitats. Still, not all species could be analysed. Analyses of isotopic composition were performed on *Ophryotrocha* sp. 1, *Ophryotrocha* sp. 2, *Ophryotrocha* sp. 3, *Ophryotrocha scutellus* and *Parougia* sp. collected from the 18 months bone samples and *Ophryotrocha* sp. 2 and *Ophryotrocha* sp. 3 from the 28 months samples.

The samples were placed in tin capsules of 8 x 5 mm and oven-dried at 60ºC for 48 hours and then sent to School of Biological Sciences in Washington State University (USA) where they were acidified with 2µl of 1N HCl with 1% $PtCl_2$ to remove inorganic carbon. Carbon and nitrogen stable isotope analyses were carried out using the EA-IRMS method (elemental analysis – isotope ratio mass spectrometry) performed on a Costech elemental analyser interfaced with a continuous-flow Micromass Isoprime isotope ratio mass spectrometer.

The C/N atomic ratios were calculated from the percentages of organic carbon and nitrogen and the isotopic composition of the samples was expressed by the relative difference between isotopic ratios in the sample and that ratios in the conventional standards as described in Chapter 2.

Imaging animals and structures: SEM – Scanning electron microscopy

Scanning electron microscopy (SEM) was used to analyse the jaw apparatus of the six *Ophryotrocha* species. To get a broad sampling of different wearing and development stages, specimens of as many different sizes as possible were used. Preparation of the jaw apparatus for SEM involved decapitating anterior ends of ethanol fixed specimens and dissociation of the soft tissue through enzymatic digestion with a 10 mg/ml dissolution of pancreatin (Macnaughton et al. 2010). Samples of soft tissue have been kept for molecular confirmation of species identification.

A total of 28 jaw apparatuses were observed and photographed using a FEI Quanta FEG 650 Scanning Electron Microscope and a NEC MultiSync PA 241w computer with the software xT Microscope Control (v 6.2.3 build 3035 – supervisor) to acquire and treat the pictures.

Although it was not possible to image *Parougia* sp. using SEM, all specimens prepared for SEM were previously observed and photographed with an automated upright Leica DM5000 B microscope. The Leica Application Suite was the software used to acquire the images.
This work was performed in the Natural History Museum, London.

Results

Dorvilleidae species density and composition

A total of 4345 individuals were collected: 237 from Bone B, the sample with the lowest number of individuals, 744 from Bone C, 3052 from Bone G, the sample with the higher number of individuals and 312 from Bone H. This totalized 981 and 3364 individuals from the samples collected 18 and 28 months after the deployment, respectively.

The highest density of dorvilleids was found in bone G (6.29 individuals.cm^{-2}) followed by bone C (2.87 individuals.cm^{-2}). Both bone B and H showed similar densities of individuals: 0.52 and 0.58 individuals.cm^{-2}, respectively. The density of individuals of the genus *Ophryotrocha* followed the same pattern (Table 3.1). In the bones B, C and H the density of dorvilleids was inversely related to that of the mytilid mussel "*Idas*" *simpsoni*; in bone G the density of dorvilleids was approximately threefold that of the mytilid mussel "*Idas*" *simpsoni* (Figure 3.3).

Table 3.1. Density of Dorvilleidae and *Ophryotrocha* spp. in each bone.

Bone samples	Bone surface area (cm^2)	Total number of Dorvilleidae	Dorvilleidae density (ind. cm^{-2})	Total number of *Ophryotrocha*	*Ophryotrocha* spp. density (ind.cm^{-2})
Bone B	459.06	237	0.52	224	0.49
Bone C	259.47	744	2.87	705	2.72
Bone G	485.34	3052	6.29	2990	6.16
Bone H	537.39	312	0.58	309	0.58

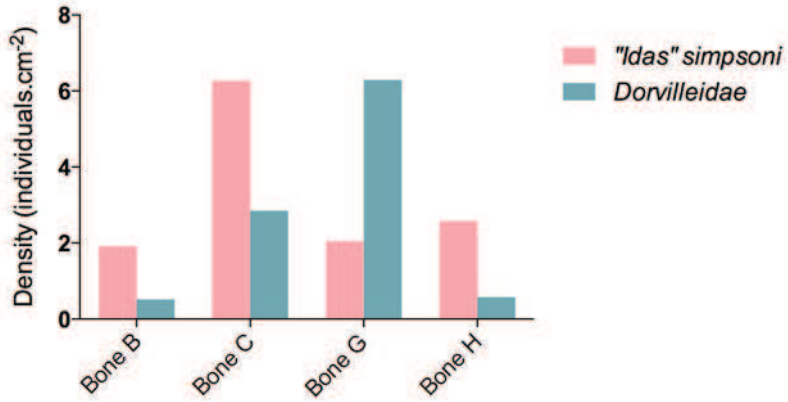

Figure 3.3. Density (individuals.cm^{-2}) of individuals of "*Idas*" *simpsoni* and Dorvilleidae in each bone sample.

Dorvilleidae composition

Species composition of all samples and putative microhabitats within the bones are shown in Table 3.2 and Figure 3.4 and Figure 3.5. All species encountered in this study were present in both sampling timepoints; bone H was the most diverse sample with all species present.

Ophryotrocha sp. 2 showed the higher number of specimens in both sampling times with 2694 adults and 309 juveniles. The second most abundant species was *Ophryotrocha* sp. 3, with 881 adults and 287 juveniles. Between the two sampling times there was an increase in the frequency of *Ophryotrocha* sp. 3, which was particularly evident in bone H (Figure 3.4), and an overall decrease in the frequency of juveniles of both *Ophryotrocha* sp. 2 and sp. 3. *Ophryotrocha* sp. 1 and *Parougia* sp. were relatively more frequent at the first sampling time (Figure 3.5). The remaining species (*Ophryotrocha* sp. 4, *Ophryotrocha* sp. 5 and O. *scutellus*) appeared in relatively small numbers and frequency in all samples.

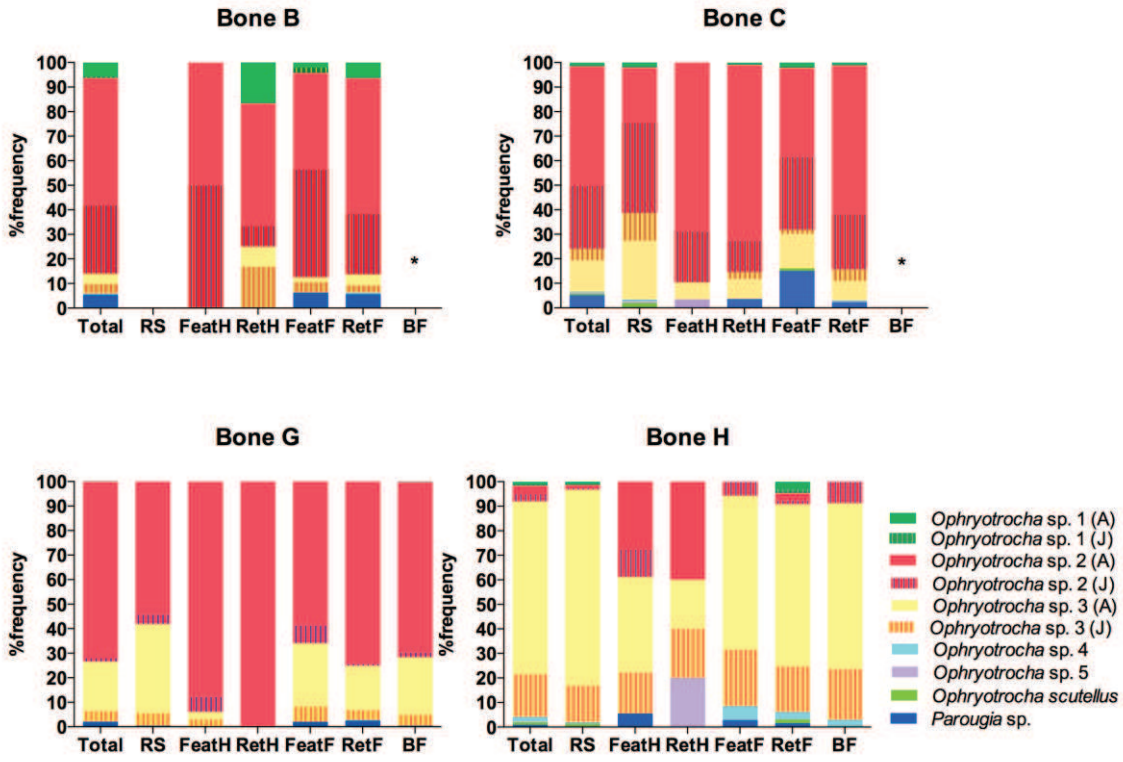

Figure 3.4. Species composition in percentage frequency of specimens of each distinct area of bone B (top, left) and bone C (top, right), collected 18 months after the deployment of the carcasses and bone G (bottom, left) and bone H (bottom, right), 28 months bone samples.
RS: Rugged and soft; FeatH: Featureless and hard surface; RetH: Reticulated and hard surface; FeatF: Featureless and friable surface; RetF: Reticulated and friable surface; BF: Filamentous bacteria (* not registered in bone B or bone C). (A): Adults; (J): Juveniles.

Table 3.2. Species composition in absolute number of specimens of each distinct area of all bone samples and the totals of each timepoint. RS: Rugged and soft; FeatH: Featureless and hard surface; RetH: Reticulated and hard surface; FeatF: Featureless and friable surface; RetF: Reticulated and friable surface; BF: Filamentous bacteria. (A): Adults; (J): Juveniles.

	Bone B						Bone C						Total 18 months	Bone G						Bone H						Total 28 months	Total
	RS	FeatH	RetH	FeatF	RetF	BF	RS	FeatH	RetH	FeatF	RetF	BF		RS	FeatH	RetH	FeatF	RetF	BF	RS	FeatH	RetH	FeatF	RetF	BF		
***Ophryotrocha* sp. 1 (A)**	0	0	2	1	11	--	3	0	1	4	3	--	25	0	0	0	0	0	1	2	0	0	0	2	0	5	30
***Ophryotrocha* sp. 1 (J)**	0	0	0	1	0	--	0	0	0	0	0	--	1	0	0	0	0	0	0	0	0	0	0	1	0	1	2
***Ophryotrocha* sp. 2 (A)**	0	1	6	19	97	--	34	20	79	70	160	--	486	130	58	1	57	1649	302	2	5	2	0	2	0	2208	2694
***Ophryotrocha* sp. 2 (J)**	0	1	1	21	43	--	55	6	14	57	59	--	257	9	4	0	7	15	8	1	2	0	2	1	3	52	309
***Ophryotrocha* sp. 3 (A)**	0	0	1	1	8	--	17	0	3	3	12	--	45	87	2	0	25	400	102	124	7	1	22	43	23	836	881
***Ophryotrocha* sp. 3 (J)**	0	0	2	2	5	--	36	2	9	27	21	--	104	12	2	0	6	90	19	23	3	1	8	12	7	183	287
***Ophryotrocha* sp. 4**	0	0	0	0	1	--	1	0	0	0	1	--	3	0	0	0	0	4	0	1	0	0	2	2	1	10	13
***Ophryotrocha* sp. 5**	0	0	0	0	0	--	1	1	0	0	1	--	3	0	0	0	0	0	0	0	0	1	0	0	0	1	4
O. scutellus	0	0	0	0	0	--	3	0	0	2	0	--	5	0	0	0	0	0	0	2	0	0	0	1	0	3	8
***Parougia* sp.**	0	0	0	3	10	--	0	0	4	29	6	--	52	1	0	0	2	57	2	0	1	0	1	1	0	65	117

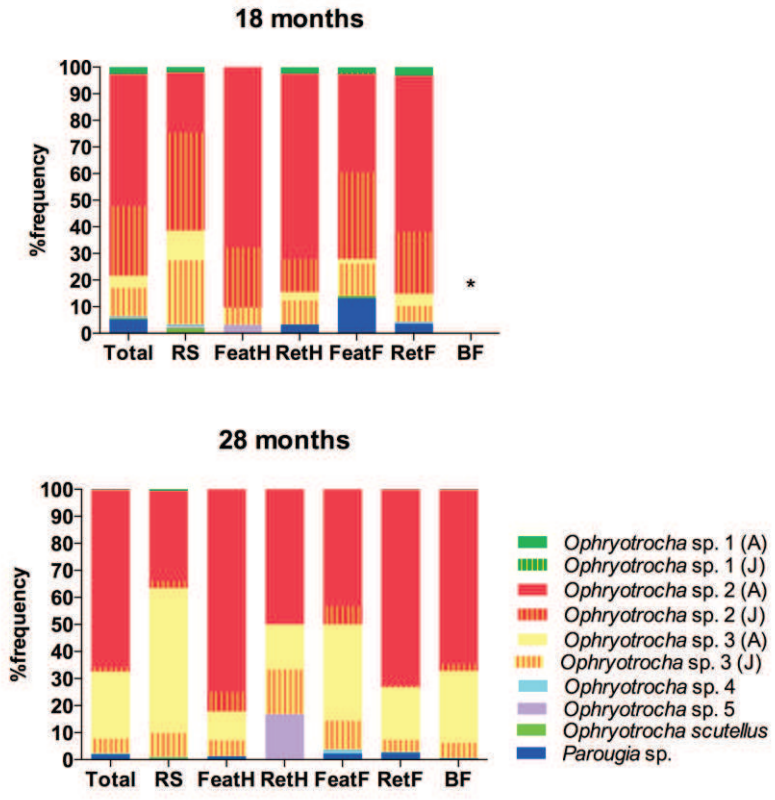

Figure 3.5. Species composition in percentage frequency of 18 months (top) and 28 months bone samples (bottom).
RS: Rugged and soft; FeatH: Featureless and hard surface; RetH: Reticulated and hard surface; FeatF: Featureless and friable surface; RetF: Reticulated and friable surface; BF: Filamentous bacteria (* not registered in bone B or bone C). (A): Adults; (J): Juveniles.

Ophryotrocha sp. 2 and *Ophryotrocha* sp. 3 also showed the highest biomass values in both sampling times (Table 3.3 and Figure 3.6). Generally, biomass contribution of the different species was similar to that of total number of individuals, reflecting the similar size of all species. In the 18 months samples *Ophryotrocha* sp. 1 had a higher contribution to the biomass of the dorvilleid community than its contribution in terms of number of individual. However this difference may be due to the fact that *Parougia* sp. was not considered in the biomass analyses.

Regarding the distribution of dorvilleids within the bones, in both timepoints the microhabitat with higher number of individuals was RetF (reticulated and friable surface), although in bone H this was not observed. In general, hard surfaces, both featureless (FetH) and reticulated (RetH) presented the lowest number of individuals. The temporal patterns described above (increase in the frequency of *Ophryotrocha* sp. 3 and decrease of juveniles) were observed in all microhabitats. Of the most abundant species, *Ophryotrocha* sp. 2 and *Ophryotrocha* sp. 3 occured in all putative microhabitats, whereas *Parougia* sp. occurred mainly in friable surfaces (RetF and FeatF), although it was rarely present in the other microhabitat with soft surface (RS). Differences in species

composition between microhabitats were mainly due to the contribution of the less abundant species (*Ophryotrocha* sp. 4, *Ophryotrocha* sp. 5 and *O. scutellus;* Table 3.2).

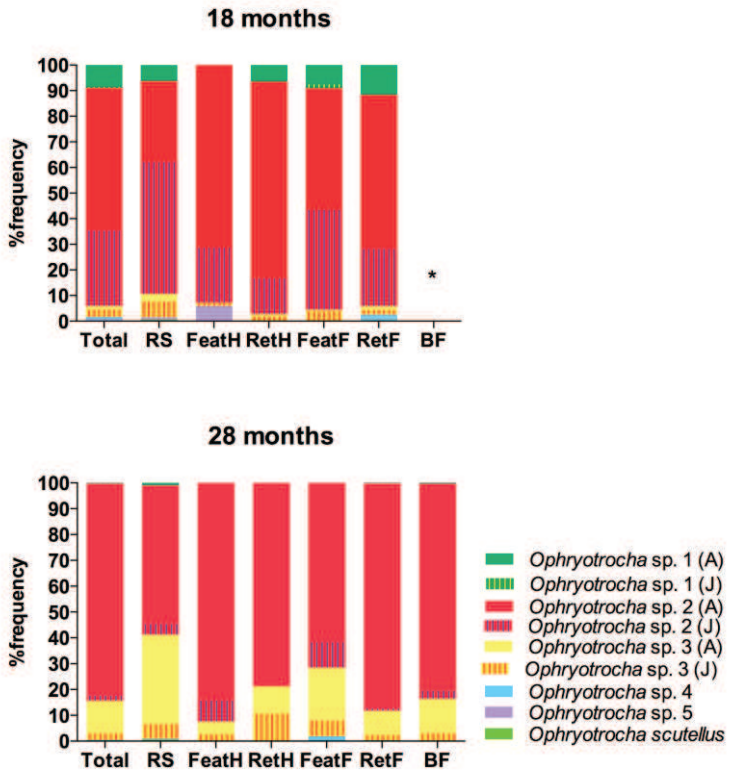

Figure 3.6. Biomass percentage frequency of 18 and 28 months samples.
RS: Rugged and soft; FeatH: Featureless and hard surface; RetH: Reticulated and hard surface; FeatF: Featureless and friable surface; RetF: Reticulated and friable surface; BF: Filamentous bacteria (* not registered in bone B or bone C). (A): Adults; (J): Juveniles.

Table 3.3. Total biomass in mg of each bone sample and species. RS: Rugged and soft; FeatH: Featureless and hard surface; RetH: Reticulated and hard surface; FeatF: Featureless and friable surface; RetF: Reticulated and friable surface; BF: Filamentous bacteria. (A): Adults; (J): Juveniles.

	Bone B						Bone C						Total 18 months
	RS	FeatH	RetH	FeatF	RetF	BF	RS	FeatH	RetH	FeatF	RetF	BF	
***Ophryotrocha* sp. 1 (A)**	0	0	0.41	0.21	2.27	--	0.62	0	0.21	0.83	0.62	--	5.17
***Ophryotrocha* sp. 1 (J)**	0	0	0	0.21	0	--	0	0	0	0	0	--	0.21
***Ophryotrocha* sp. 2 (A)**	0	0.09	0.56	1.76	8.99	--	3.15	1.85	7.32	6.49	14.83	--	45.04
***Ophryotrocha* sp. 2 (J)**	0	0.09	0.09	1.95	3.99	--	5.10	0.56	1.30	5.28	5.47	--	23.83
***Ophryotrocha* sp. 3 (A)**	0	0	0.02	0.02	0.14	--	0.29	0	0.05	0.05	0.21	--	0.78
***Ophryotrocha* sp. 3 (J)**	0	0	0.03	0.03	0.09	--	0.62	0.03	0.16	0.47	0.36	--	1.79
***Ophryotrocha* sp. 4**	0	0	0	0	0.63	--	0	0	0	0	0	--	0.63
***Ophryotrocha* sp. 5**	0	0	0	0	0	--	0.08	0.15	0	0	0	--	0.23
O. scutellus	0	0	0	0	0	--	0.06	0	0	0.04	0	--	0.10

	Bone G						Bone H						Total 28 months
	RS	FeatH	RetH	FeatF	RetF	BF	RS	FeatH	RetH	FeatF	RetF	BF	
***Ophryotrocha* sp. 1 (A)**	0	0	0	0	0	0.22	0.45	0	0	0	0.45	0	1.12
***Ophryotrocha* sp. 1 (J)**	0	0	0	0	0	0	0	0	0	0	0.22	0	0.22
***Ophryotrocha* sp. 2 (A)**	24.69	11.02	0.19	10.82	313.22	57.36	0.38	0.95	0.38	0	0.38	0	419.39
***Ophryotrocha* sp. 2 (J)**	1.71	0.76	0	1.33	2.85	1.52	0.19	0.38	0	0.38	0.19	0.57	9.88
***Ophryotrocha* sp. 3 (A)**	6.66	0.15	0	1.91	30.61	7.80	9.49	0.54	0.08	1.68	3.29	1.76	63.97
***Ophryotrocha* sp. 3 (J)**	0.92	0.15	0	0.46	6.89	1.45	1.76	0.23	0.08	0.61	0.54	0.54	13.63
***Ophryotrocha* sp. 4**	0	0	0	0	0.64	0	0.16	0	0	0.32	0.32	0.16	1.60
***Ophryotrocha* sp. 5**	0	0	0	0	0	0	0	0	0	0	0	0	0
O. scutellus	0	0	0	0	0	0	0.24	0	0	0	0.12	0	0.36

Multidimensional scaling (MDS) analyses performed to compare the species composition between different microhabitats in each sampling time showed segregation in three groups in the samples collected 18 months after the deployment (Figure 3.7): 1) featureless and friable (FeatF) and reticulated and hard (RetH) surfaces exhibited a similar species composition; 2) Reticulated and friable (RetF) and rugged and soft (RS) also showed similarity, although with a higher degree of dispersion between them; and 3) featureless and hard (FeatH) microhabitat presented the most different dorvilleid species composition at this sampling time. The MDS analyses of the 28 month samples showed low segregation, with the reticulated and hard surface (RetH) presenting the most distinct dorvilleid species composition, and rugged and soft surface (RS) and surface covered by filamentous bacteria (BF) the most similar (Figure 3.8).

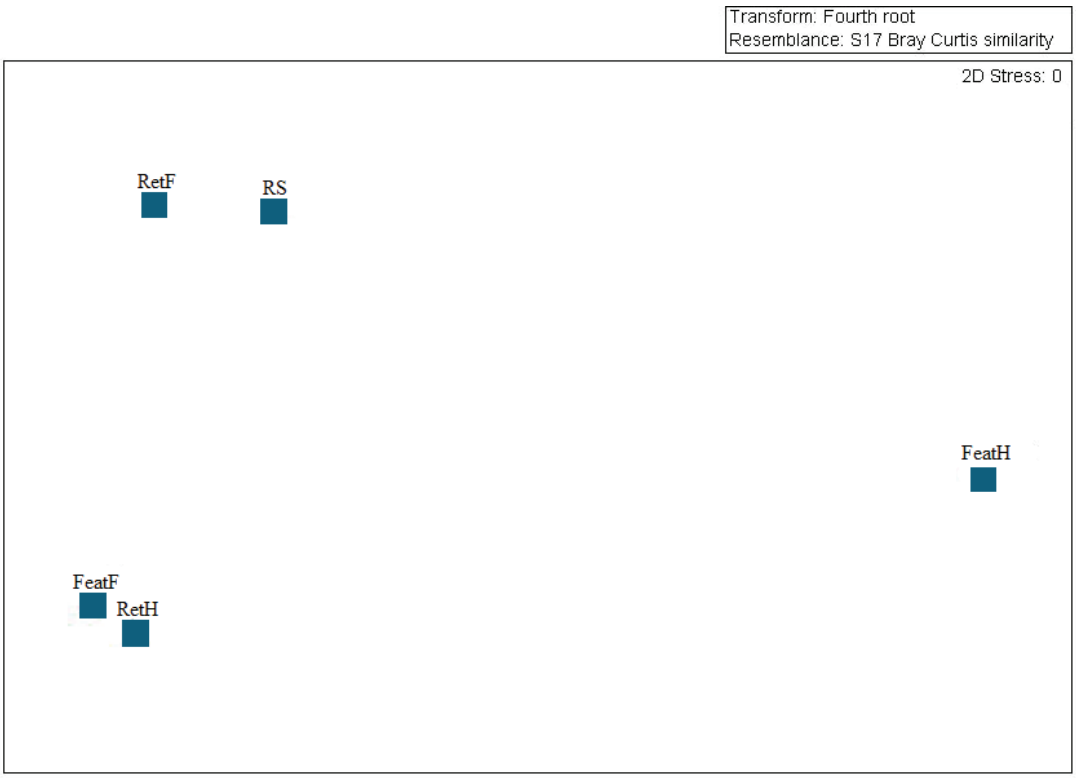

Figure 3.7. MDS plot of the composition of Dorvilleids in different microhabitats of the 18 months bone samples after a fourth root transformation.
RS: Rugged and soft; FeatH: Featureless and hard surface; RetH: Reticulated and hard surface; FeatF: Featureless and friable surface; RetF: Reticulated and friable surface.

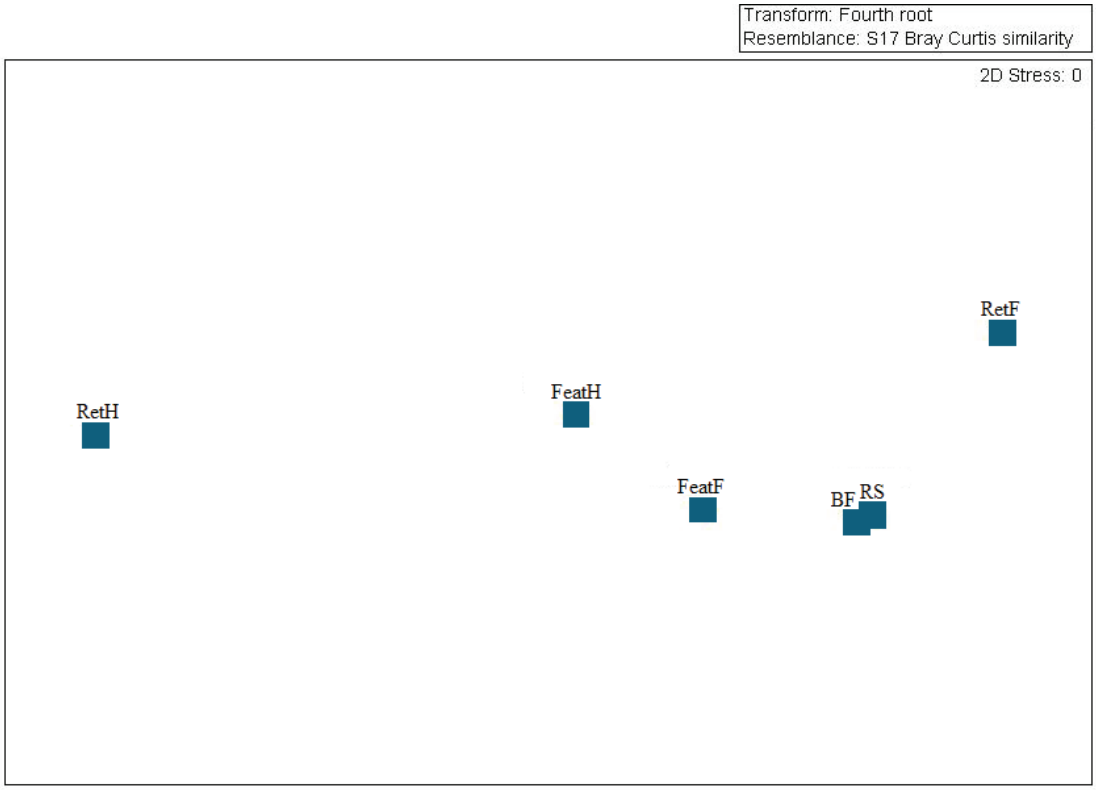

Figure 3.8. MDS plot of the composition of Dorvilleids in different microhabitats of the 28 months bone samples after a fourth root transformation.
RS: Rugged and soft; FeatH: Featureless and hard surface; RetH: Reticulated and hard surface; FeatF: Featureless and friable surface; RetF: Reticulated and friable surface; BF: Filamentous bacteria.

Isotopic signatures

Stable isotope analyses showed that different dorvilleid species exhibited distinct carbon and nitrogen isotopic signatures (Figure 3.9). Carbon isotope values ranged from -26.1 in *Ophryotrocha* sp. 3 from the 28 months samples to -24.6 in *O. scutellus*. *Ophryotrocha* sp. 3 (28 months) also exhibited the highest $\delta^{15}N$ value (8.6) whereas *Ophryotrocha* sp. 2 from the 18 months samples showed the lowest (4.4). Although it was not possible to perform statistical analyses three groups of isotopic signatures could be distinguished: 1) *Ophryotrocha* sp. 3 from 28 months; 2) *Ophryotrocha* sp. 1 and *O. scutellus* and 3) *Ophryotrocha* sp. 2 from both sampling times and *Ophryotrocha* sp. 3 from 18 months and *Parougia* sp.. Analyses of the bones presented a mean $\delta^{13}C$ value of -21.8 and a mean $\delta^{15}N$ value of 6.2.

The isotopic values found in this study are within the range of those encountered in previous studies performed in species from deep-sea chemosynthetic environments (Table 3.4).

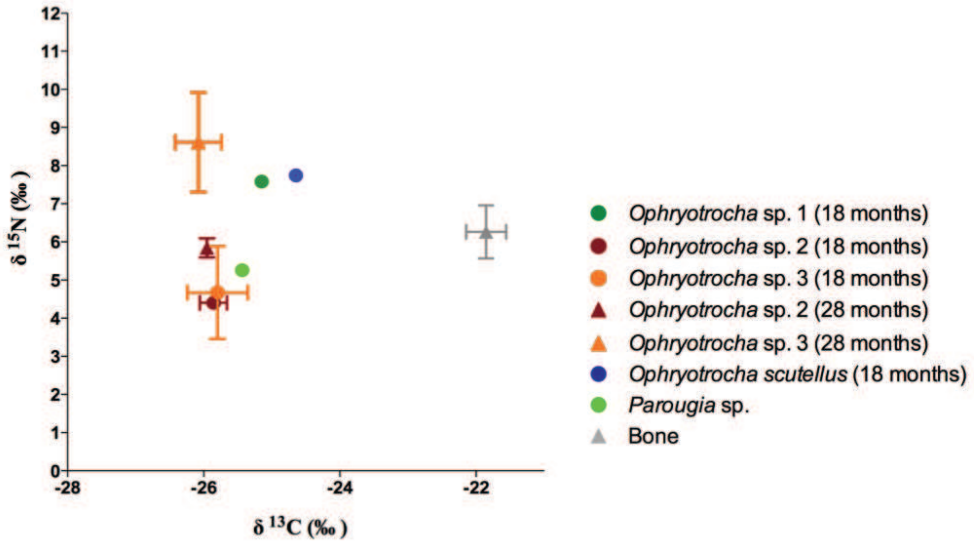

Figure 3.9. Stable isotope values for the distinct *Ophryotrocha* species in different timepoints and for the bones used to perform this study. Colors and symbols indicate distinct species, different sampling times and the stable isotope values for the bones. *Ophryotrocha* sp. 3, *Ophryotrocha* sp. 2, *Ophryotrocha* sp. 1, *O. scutellus* and *Parougia* sp. were collected from 18 months samples. 28m correspond to the isotopic signature of individuals collected in 28 months bone samples. Bone represents a mean value for isotopic signature of bone C and H.

Table 3.4. List of Dorvilleidae species for which isotopic composition has been studied.

Species	Habitat	Site	Depth	$\delta^{13}C$	$\delta^{15}N$	Reference
Dorvilleidae	Organic fall	NE Atlantic	1004	(-26.1 to -24.6)	8.6 to 4.4	This study
Ophryotrocha maciolekae	Monocultures			-24.5		(Thurber et al. 2012)
Ophryotrocha platykephale	Monocultures			-26.2		(Thurber et al. 2012)
Parougia sp.	Monocultures			-39.4		(Thurber et al. 2012)
Dorvillea sp.	Monocultures			-91.5		(Thurber et al. 2012)
Dorvilleidae	Cold seep	Norwegian sea		-48.4	1.4	(Decker and Olu 2011)
Dorvilleidae	Cold seep	Norwegian sea		-48.3	1.4	(Decker and Olu 2010)
Dorvilleidae	Cold seep	NZ margin	216	-29.7	-1.0	(Thurber et al. 2009)
Dorvilleidae	Cold seep	NZ margin	197	-26.0		(Thurber et al. 2009)
Dorvilleidae	Cold seep	NZ margin	86	-29.0		(Thurber et al. 2009)
Dorvilleidae	Cold seep	NZ margin	277	-25.0		(Thurber et al. 2009)
Dorvilleidae	Cold seep	Mediterranean	Dive 334	-22.9	5.0	(Carlier et al. 2010)
Ophryotrocha globopalpata	Hydrothermal vent	NE Pacific	Dive 3138	-25.0	– 5.6	(Bergquist et al. 2007)
Dorvilleidae	Cold seep	Pacific Ocean		-90.6	7.5	(Levin and Mendoza 2007)
Dorvilleidae und.	Cold seep	Pacific Ocean		-33.8	-0.5	(Levin and Mendoza 2007)
Dorvilleidae	Cold seep	NE Pacific Ocean	520	-38.1	5.7	(Levin and Michener 2002)
Dorvilleidae	Cold seep	NE Pacific Ocean	520	-73.8	5.7	(Levin and Michener 2002)
Dorvilleidae	Cold seep	NE Pacific Ocean	520	-22.4	13.9	(Levin and Michener 2002)
Dorvilleidae	Cold seep	NE Pacific Ocean	520	-21.4	11.4	(Levin and Michener 2002)
Dorvilleidae	Cold seep	NE Pacific Ocean	520	-21.7	11.5	(Levin and Michener 2002)
Dorvilleidae sp. A	Cold seep	NE Pacific Ocean	520	-22.1	9	(Levin and Michener 2002)
Dorvilleidae sp. A	Cold seep	NE Pacific Ocean	520	-20.5	10.0	(Levin and Michener 2002)
Dorvilleidae sp. A	Cold seep	NE Pacific Ocean	520	-22.2	11.9	(Levin and Michener 2002)
Dorvilleidae sp. B	Cold seep	NE Pacific Ocean	520	-35.9	12.3	(Levin and Michener 2002)
Dorvilleidae sp. C	Cold seep	NE Pacific Ocean	520	-27.0	8.0	(Levin and Michener 2002)

Morphology of the jaw apparatus

Figure 3.10 shows the general morphology of the jaw apparatus of the different dorvilleid species encountered in this study. Both adults and juveniles of *Ophryotrocha* sp. 1 showed rod-like mandibles with U-shaped cutting plates exhibiting 2 anterior peaks; the maxillae was type P and comb-like. *Ophryotrocha* sp. 2 (adults and juveniles) presented rod-like mandibles with cutting plates and apophyses greatly extending from median lateral edge of the cutting plates. The P-type maxillae exhibited basal plates with variable number of teeth. Juveniles and adults of *Ophryotrocha* sp. 3 showed some differences in the jaw apparatus: juveniles exhibited a jaw apparatus similar to *Ophryotrocha* sp. 2 whereas adults presented a more robust structure. Adults of *Ophryotrocha* sp. 3 exhibited rod-like and robust mandibles with cutting plates with a worn light serration; usually exhibited P-type maxillae with basal plates comb-like with variable number of teeth.

Ophryotrocha sp. 4 presented a mandible with two lateral external peaks and a serration on the top of the cutting plates. The P-type maxillae showed to be similar to *Ophryotrocha* sp. 3 with basal plates comb-like with variable number of teeth. *Ophryotrocha* sp. 5 robust mandibles exhibited cutting plates with a worn ligh serration with no peaks and the P-type maxillae showed a comb-like structure. Generally, the jaw apparatus of this species was similar to that of adults *Ophryotrocha* sp. 3. *Ophryotrocha scutellus* showed a jaw apparatus similar to *Ophryotrocha* sp. 1. *Parougia* sp. exhibited a delicate mandible with cutting plates presenting a clear serration. The P-type maxillae exhibited basal plates with variable number of teeth.

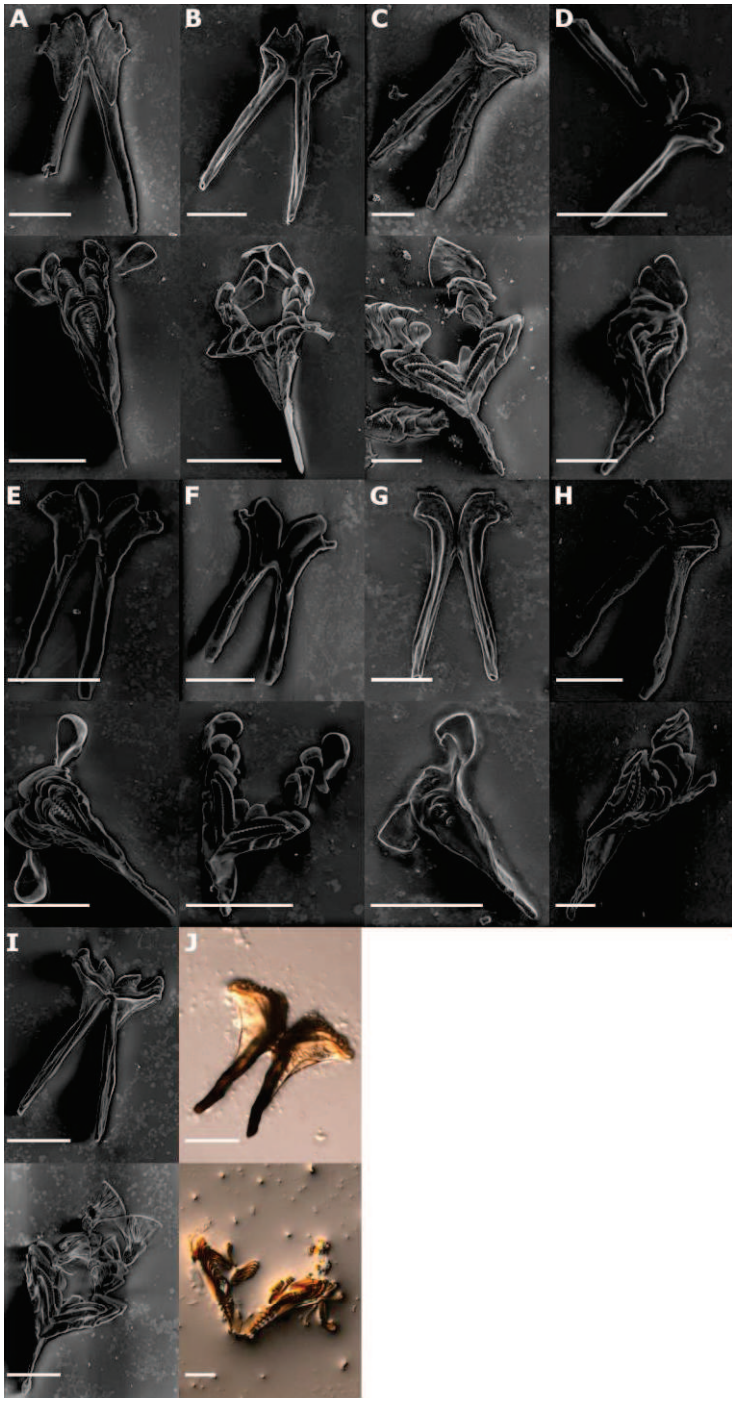

Figure 3.10. SEM images of Dorvilleidae species. A: Mandible (top) and maxillae (bottom) of an adult of *Ophryotrocha* sp. 1 (scale bar – mandible: 50 µm; maxillae: 100 µm); B: Mandible (top) and maxillae (bottom) of a juvenile of *Ophryotrocha* sp. 1 (scale bar – mandible: 50 µm; maxillae: 100 µm); C: Mandible (top) and maxillae (bottom) of an adult of *Ophryotrocha* sp. 2 (scale bar – mandible: 50 µm; maxillae: 50 µm); D: Mandible (top) and maxillae (bottom) of a juvenile of *Ophryotrocha* sp. 2 (scale bar – mandible: 50 µm; maxillae: 40 µm); E: Mandible (top) and maxillae (bottom) of an adult of *Ophryotrocha* sp. 3 (scale bar – mandible: 50 µm; maxillae: 50 µm); F: Mandible (top) and maxillae (bottom) of a juvenile of *Ophryotrocha* sp. 3 (scale bar – mandible: 30 µm; maxillae: 50 µm); G: Mandible (top) and maxillae (bottom) of *Ophryotrocha* sp. 4 (scale bar – mandible: 50 µm; maxillae: 100 µm); H: Mandible (top) and maxillae (bottom) of *Ophryotrocha* sp. 5 (scale bar – mandible: 50 µm; maxillae: 50 µm); I: Mandible (top) and maxillae (bottom) of *O. scutellus* (scale bar – mandible: 50 µm; maxillae: 50 µm); J: Mandible (top) and maxillae (bottom) of *Parougia* sp. (scale bar – mandible: 50 µm; maxillae: 50 µm).

Discussion

Temporal variation in the Dorvilleidae assemblage

One of the goals of this study was to understand the temporal patterns of colonisation in the bones collected from the carcasses deployed in the Setubal canyon. For that, the density and composition of the dorvilleid assemblages present in four bones collected at two different times were analysed.

The dorvilleid assemblages collected from the bones herein analysed showed values ranging from 0.52 to 6.29 individuals.cm^{-2} and generally higher than those reported for assemblages from other deep-sea chemosynthetic environment, particularly cold seeps in the Northern California margin in which densities varied from 0.07 to 1.10 ind.cm^{-2} (Levin et al. 2003). The variation found between samples in this study does not seem to be temporal but rather related to different conditions, either chemical or biological, presented by the different bones. In bone B, C and H, dorvilleid density was inversely related to the density of "*Idas*" *simpsoni* showing a ratio of approximately 1:2. In bone G the opposite occurred with the density of dorvilleid worms being approximately threefold that of "*I.*" *simpsoni*. As already referred in Chapter 2, this range of values may be due to differences in the chemical composition of the bone or result from ecological interactions. The chemical composition of the different bones were not analysed in this study, and therefore it is not possible to establish a relationship between the densities of invertebrates and a possible preference by the chemical composition of the substrate. Mussels and dorvilleids densities may be explained by inter-specific competition for space. Dorvilleid worms are known to be scavengers or microbial grazers (Fauchald et al. 1979, Word 1979) and therefore their nutritional sources (bone/meat or microbial mats) are not available in areas occupied by mussels. Nevertheless, the whole invertebrate community of each bone was not investigated and thus other inter-specific interactions may occur.

Regarding species composition over the two different sampling times, notorious differences were observed. Although *Ophryotrocha* sp. 2 was the dominant species on both sampling times, the 28 months samples showed a clear increase in the total number and frequency of *Ophryotrocha* sp. 3. The total number of *Ophryotrocha* sp. 4, *Ophryotrocha* sp. 5 and *O. scutellus* were also higher on the sampling time, whereas those of *Ophryotrocha* sp. 1 and *Parougia* sp. decreased. These differences in dorvilleid composition may be related to the presence of specific food sources (Levin et al. 2003, 2013), such as microbial mats, that occur in different stages of bone degradation. For example, it has been described that the growth and maintenance in the deep sea of filamentous bacteria, which were only found in the second sampling time, depend on the oxidation of inorganic compounds (Schopf and Klein 1992).

An evident reduction of the percentage of juveniles on the second sampling time was also observed. However, the lack of data on an intermediate stage of colonisation and on the life-cycle of the dorvilleid species hereby analysed does not allow to infer if the adults present on the 28 months samples are a result of the development of the high number of juveniles present 10 months before.

Species distribution within the bones

The co-occurrence of several dorvilleid species in deep-sea chemosynthetic environments has been explained as a result of niche partitioning through trophic patterns and habitat preference/tolerance (Levin et al. 2003, 2013). The bones collected during CARCACE project exhibited visually distinct surface patches, with different textures, hardness and coloration, that may represent different microhabitats within each bone. Having this in consideration, six different putative microhabitats were defined and their species composition was analysed.

Although the analyses of the species composition of the different microhabitats showed some spatial patterns, such as fewer individuals on hard surfaces and the higher frequency of *Parougia* sp. in friable areas, the multidimensional scaling (MDS) analyses showed no segregation according to the features used to characterise the microhabitats: texture, hardness and conspicuous presence of filamentous bacteria. Therefore, the observed segregation showed by the MDS analyses must be related to other, non-considered, substrate characteristics that may be especially relevant for the presence of the more rare species (*Ophryotrocha* sp. 4, *Ophryotrocha* sp. 5 and *O. scutellus*), since these have a major contribution for the differences in species composition between microhabitats.

Nutritional sources and jaw morphology

In order to investigate whether different dorvilleid species use distinct nutritional sources, stable isotopic analyses were performed. The obtained isotopic signatures were in the range of previous values obtained for dorvilleid polychaetes from deep-sea chemosynthetic environments, suggesting a chemosynthesis-based nutrition of the species inhabiting the bones from the carcasses deployed in the Setubal canyon. Isotopic signatures of dorvilleids associated with specific methane seepage microhabitats (clambeds or microbial mats) are usually more negative, ranging from -91.5 to -48.3 but these refer to other genus of Dorvilleidae family different from *Ophryotrocha* or *Parougia* (Table 3.4).

Different isotopic compositions indicate different food sources (Sulzman 2007), and in the particular case of deep-sea dorvilleid polychaetes may indicate the consumption of different bacteria (Levin and Michener 2002). Within the studied species three groups of signatures were distinguished: 1) *Ophryotrocha* sp. 3 from 28 months; 2) *Ophryotrocha* sp. 1 and *O. scutellus* and

3) *Ophryotrocha* sp. 2 from both sampling times and *Ophryotrocha* sp. 3 from 18 months and *Parougia* sp..The difference in the isotopic signatures of *Ophryotrocha* sp. 3 from the two sampling times may result from differences in the development stage of the individuals: the majority of the individuals collected at 18 months were juveniles, whereas the individuals collected at 28 months were all adults.

The jaw apparatuses are important diagnostic features in Dorvilleidae (Purschke 1987) but their role in the trophic ecology has never been studied. The analyses of the jaw apparatus of the studied species revealed different morphologies, particularly in the mandible, that can be related to their isotopic signatures:

1. Adult *Ophryotrocha* sp. 3 (from the bones collected after 28 months) exhibited rod-like and robust mandibles with cutting plates with a worn light serration and usually exhibit P-type maxillae with basal plates comb-like with variable number of teeth.

2. *Ophryotrocha* sp. 1 and *O. scutellus* showed the most robust rod-like mandibles of all species, and have U-shape cutting plates with 2 anterior peaks. Both species exhibit P-type comb-like maxillae.

3. *Ophryotrocha* sp. 2, juvenile *Ophryotrocha* sp. 3 and *Parougia* sp. presented a delicate mandible and the P-type maxillae exhibited basal plates with variable number of teeth.

Differences in the morphology of the jaw apparatus may therefore allow the exploitation of different food sources corroborating the idea that multiple species can co-occur by nutritional resource partitioning (Levin et al. 2003, 2013).

Conclusion

Polychaetes of the family Dorvilleidae are important members of deep-sea reduced habitats, particularly organic-falls (Bernardino et al. 2010, 2012, Wiklund et al. 2012). This is the first study analysing ecological aspects of the Dorvilleidae assemblages associated with mammal carcasses in the deep northeast Atlantic, where these habitats remain poorly investigated (Hilário et al. (*in press*)).

The analyses of the bones from the carcasses deployed in the Setubal canyon revealed high abundances and densities of dorvilleid worms, as well as a high number of species. The species composition varied temporally possibly reflecting the availability of different food source at different degradation stages. Species composition in different microhabitats showed that dorvilleid distribution within the bones is not related to the texture, hardness and presence of filamentous bacteria on the surface of the bone, but rather to other substrate features that could not be identified in the timeframe of this study. For example, it would be interesting to analyse and describe the microbial community, thus the potential food sources, associated to each microhabitat.

The exploitation of different nutritional resources by different species was evinced by isotopic analyses that disclosed three main groups of dorvilleid species. These groups showed different morphology of the chitonized jaw apparatus indicating for the first time the potential importance of this structure in the specialisation of dorvilleids on distinct food sources, and consequently on their trophic ecology.

CHAPTER 4. GENERAL CONCLUSIONS

Mammal carcasses at the deep-sea floor provide a resource for unique and highly diverse faunal assemblages that include generalist-scavenging species, chemosynthetic fauna and bone specialists. In the last two decades, deep-sea researchers have studied the colonisation of dozens of sunken whale carcasses, both natural and experimentally implanted. However, these studies have been restricted to the Pacific Ocean. The CARCACE project aimed to investigate for the first time the community response to an intense pulse of organic material in the deep-Atlantic Ocean and for that, mammal carcasses were deployed at 1000 m depth in the Setubal canyon. Analyses of the invertebrate assemblages found in the bones resulting from the degradation of the carcasses deployed in the Setubal canyon revealed high species and trophic diversity. However, temporal and spatial (within bone) patterns of colonisation were not studied and it was in this context that the present thesis was developed.

The first goal of this study was to investigate the settlement patterns and possible temporal shifts in the feeding strategy (filter feeding *versus* chemosymbiosis) of the bivalve "*Idas*" *simpsoni*, a chemosymbiotic mussel known mainly from cetacean bones but also found on carbonates near seeps (Chapter 2). Analyses of the population size structure disclosed continuous settlement and a limitation in growth and adult survival. It is hypothesised that these restrictions might be due to insufficient energy supplied by the cow bone in comparison with other reducing habitats, which is supported by the isotopic signatures of "*Idas*" *simpsoni* that indicated a higher contribution of filter feeding to their nutrition. However, intraspecific competition for food and space may also contribute to the reduced size of the individuals. Post-settlement density-dependent mortality of adults with the likely survival of a small number of females affect the population structure, but effective recruitment may still be possible contributing to the dispersal of the species. This study provides evidence on the role of organic falls to bridge dispersal between reducing habitats and highlights the importance of these substrates on biogeographic distribution of chemosymbiotic species.

The second goal was to investigate the spatial and temporal colonisation patterns of polychaetes from the family Dorvilleidae (Chapter 3). Dorvilleids are opportunistic scavengers or microbial grazers that are frequently dominant in polluted and eutrophic sediments but also occur in deep-sea reduced habitats, including whale-falls. In the CARCACE assemblages, at both sampling times, dorvilleids were one of the most abundant taxa and the most diverse, with seven different species, 6 of which new to science. Previous studies have suggested that dorvilleid species co-exist in deep-sea chemosynthetic habitats by niche partitioning through trophic patterns and habitat preference/tolerance. To test this hypothesis the species composition over time, their distribution

within bones and the nutritional patters of the Dorvilleidae assemblages were investigated. Clear differences in species composition were observed between the two sampling times, most likely reflecting the availability of distinct food sources at different degradation stages, since different species showed different isotopic signatures. Although different putative microhabitats within the bones could be visually identified, the species composition did not show any relation with their characteristics, namely texture, hardness and presence of filamentous bacteria on the surface. Thus, the distribution of the dorvilleid species within the bone may be related with features not considered in this study, such as the presence of different microbial mats or local chemical composition.

The use of a computerized tomography scanner to accurately measure the areas of the bones allowed to calculate the densities of "*I.*" *simpsoni* and dorvilleids, which were inversely related, possibly indicating inter-specific competition for space. Dorvilleid worms are known to be scavengers or microbial grazers thus their nutritional sources (bone/meat or microbial mats) are not available, or cannot be reached in areas occupied by mussels. Nevertheless, the whole invertebrate community of each bone was not investigated and thus other inter-specific interactions might also occur.

As part of the study of the dorvilleid assemblages, the morphology of the jaw apparatus was investigated in the context of the trophic ecology of each species. It is the first time that this important taxonomic character is analysed in an ecological perspective and results suggested a possible specialisation of dorvilleids on distinct food sources according to the morphology of this structure. However, a more detailed analysis, using more species, preferably from different habitats, is needed to fully understand the relationship between the morphology of the jaw and the trophic ecology, habitat affiliation and specialisation on different resources.

In summary, this thesis presents the most complete work on the colonisation of mammal-falls in the deep Atlantic Ocean. Classic tools like optical and electron scanning microscopy and isotopical analyses were complemented with novel approaches like tomographic imaging to measure the areas of the bones and the adaptation of an indirect method to measure the biomass of polychaetes. It provides significant insights about the ecology of the studied species and the mammal-falls in general and will set ground for further investigations.

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ANNEX

marine ecology

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SPECIAL TOPIC

Mammal carcasses attract a swarm of mussels in the deep Atlantic: insights into colonization and biogeography of a chemosymbiotic species

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Abstract

Five mammal carcasses were experimentally deployed at 1000 m depth in the Setubal Canyon (NE Atlantic) in March 2011 and the remaining bones were collected after 18 and 28 months. High numbers (1.92–6.27 individuals·cm^2) of small mussels were found among a diverse invertebrate assemblage colonizing surfaces, crevices and cavities in the trabecular matrix of bones. In this study we characterized the mussel population and their associated bacteria and investigated population structure and settlement patterns. The results of molecular analysis indicated that the mussels are conspecific with the widely distributed bathymodiolin species '*Idas*' *simpsoni* and harbor sulfur-oxidizing bacterial symbionts. Modal decomposition of length-frequency distributions and estimated shell growth rate suggested that settlement of '*I.*' *simpsoni* is continuous and that post-settlement mortality affects the population structure. This study reports the highest densities and fastest growth rates within bathymodiolin mussels occurring on organic substrates yet recorded and provides evidence for effective mussel recruitment in organic falls in the NE Atlantic Ocean. These results highlight the importance of ephemeral habitats on the biogeographic distribution and dispersal of chemosymbiotic species in deep-sea ecosystems.

Introduction

Surprisingly high abundances and a remarkable diversity of invertebrates have been observed in organically enriched habitats in the deep sea. Wood and bone fall communities share a number of taxa with cold seep and hydrothermal vent fauna, which is relevant to the hypothesis that large food falls might serve as stepping stones for dispersal among various chemosynthesis-based ecosystems (Smith *et al.* 1989; Distel *et al.* 2000; Kiel & Goedert 2006). However, the capability of organic falls to bridge dispersal among such assemblages and so ensure present-day population connectivity of their species is still being debated (Cunha *et al.* 2013). Colonization and population dynamics of discrete resource patches, varying in time and space, are far from being understood. The number of competent larvae reaching a site, the probability of successful settlement, and levels of mortality occurring over successive life-history stages determine variability in the number of individuals reaching reproductive maturity, thus affecting connectivity at different temporal and spatial scales (Kelly & Metaxas 2008).

The study of demographic patterns is hampered by technological and financial constraints to acquiring long-term data in the deep sea, and life-history strategies are only known for a relatively small number of taxa (Tyler & Young 1999). Due to their widespread distribution and high abundance in chemosynthetic habitats, mytilid bivalves are one of the best-known groups among deep-sea macro-invertebrates. More than 30 species have been

described and a large number of unidentified molecular lineages await formal description (Thubaut *et al.* 2013a). The ecological success of mussels in chemosynthetic environments is attributed to their nutritional strategy, which involves symbiotic relationships with one or several types of bacteria while maintaining filter-feeding capability, and to long-lived planktotrophic larvae capable of dispersing over large distances (Arellano & Young 2009; Duperron 2010). Several lineages of mussels have diversified in chemosynthesis-based ecosystems since the Late Cretaceous, and habitat transitions from organic substrates to vents and seeps were accompanied with physiological and morphologic adaptations (Lorion *et al.* 2013).

Life-history strategies, settlement patterns and feeding modes (including associated bacteria) are known for larger vent and seep mussels (*e.g.* Dixon *et al.* 2006; Tyler *et al.* 2007; Arellano & Young 2009; Duperron 2010), and for smaller mussels associated with organic falls (Deming *et al.* 1997; Duperron *et al.* 2008a, 2009; Tyler *et al.* 2009; Fujiwara *et al.* 2010; Thubaut *et al.* 2013b), but these latter studies were focused on the Pacific species: *Idas washingtonius* (Bernard, 1978), *Idas iwaotakii* (Habe, 1958), two undescribed *Idas* species [evolutionarily significant unit (ESU) D and ESU J], *Adipicola pacifica* (Dall, Bartsch & Rehder, 1938) and *Adipicola longissima* (Thiele & Jaeckel, 1931). From the 13 mytilid taxa reported on organically enriched substrates in the Atlantic, only *Idas modiolaeformis* (Sturany, 1896) and *Idas argenteus* Jeffreys, 1876, have been investigated (Dean 1993; Duperron *et al.* 2008b; Ockelmann & Dinesen 2011; Gaudron *et al.* 2012), but neither species was found to occur on bone substrates.

The presence of high numbers of small mussels on bones recovered from an experimental deployment of mammal carcasses in Setubal Canyon provided an opportunity to study colonization patterns over two exposure periods. The main goal of this study was to characterize the mussel population associated with the mammal bones. The specific goals were: (i) to assess the taxonomic identification of the specimens collected over the two sampling periods and describe their associated bacteria and (ii) to investigate the population size-structure and settlement patterns. Overall, this study aimed to contribute to the knowledge of life-history traits of smaller mussel species associated with mammal falls in the Atlantic Ocean.

Material and Methods

Sample collection

Mytilid mussels (Fig. 1) were collected from bones resulting from the experimental deployment of five cow carcasses at Setubal Canyon (38°16.85′N, 09°06.68′W; 1000 m depth), NE Atlantic. Details of the experiment are described in Hilário *et al.* (this issue). Exposed bones were collected approximately 18 and 28 months after deployment with the manipulator arm of ROV *Luso* and ROV *Genesis*, respectively, and brought to the ship inside closed boxes with ambient seawater, preventing dislodgment of attached fauna during transport. Bones were preserved individually onboard in 96% ethanol or in 4% seawater-formalin for DNA sequencing and morphologic analyses of colonizing organisms. In the laboratory, mussels were removed with forceps from the surfaces of four bones (Table 1) under a dissecting microscope fitted with an ocular micrometer for shell measurements. A total of 29 mussels (18 from the first sampling occasion and 11 from the second) was identified using molecular methods (see below). After the removal of mussels and other visible epifauna, the bones were imaged using a computerized tomography scanner (General Electric BrightSpeed 16) at the Hospital Infante D. Pedro (Aveiro, Portugal). Image acquisition was performed in helicoid mode with sections of 0.625 mm thickness, reconstruction interval of 0.310 mm, 100 kVp, 100 mAs, pitch of 0.562:1, and reconstruction algorithms Standard and Bone.

Molecular characterization of mussels and associated bacteria

Mussels were dissected and DNA was extracted from soft tissue using a DNeasy kit (Qiagen, Valencia, CA). A fragment of the gene encoding mitochondrial cytochrome c oxidase subunit I (mtCOI) was amplified using primers 1560F and 2148R and the conditions described in Jones *et al.* (2006). Fragments of bacterial genes encoding 16S

Fig. 1. Clump of live mussels settled on a cow bone from the experimental mammal fall in Setubal Canyon, NE Atlantic. Scale bar = 1 mm.

Table 1. Details of samples used in this study.

Sampling date	Deployment length	Bone samples	Bone surface area (cm^2)	Mussel population sample size (n)	Mussel density (individuals cm^{-2})
22.08.2012	18 months	Bone B	459.06	883	1.92
		Bone C	259.47	1628	6.27
12.06.2013	28 months	Bone G	485.34	995	2.05
		Bone H	537.39	1389	2.58

ribosomal RNA (16S rRNA) and adenosine-5′-phosphosulfate (APS) reductase were amplified for three mussel specimens collected from the first recovered bones using PCR primers and the conditions described in Rodrigues *et al.* (2013). For the 16S rRNA gene, PCR products were purified, cloned using a TOPO TA cloning kit (Invitrogen, CA) and inserts from 80 positive clones were sequenced. For the gene encoding APS reductase, PCR products were sequenced directly and, as the sequence displayed no ambiguities such as double peaks, no additional cloning step was added.

Sequences were compared with those on the GenBank database using BLAST and deposited in this database under accession numbers HG931850−879. Sequence alignments including BLAST hits and representative sequences were generated for the mtCOI gene with ClustalW and edited manually (Thompson *et al.* 1997). To estimate the relationships among haplotypes, a median network using the median-joining algorithm was generated in the program SPLITS TREE v.4 (Huson & Bryant 2006). Kimura two-parameter (K2P) genetic distances among sequences were calculated with MEGA v.5.2 (Kimura 1980; Tamura *et al.* 2011).

Data analyses

The imaging software 3D SLICER (http://www.slicer.org/) was used on bone-reconstructed images to estimate the area of water-exposed surface. This area was used to calculate the density of individuals (individuals·cm^{-2}) on each bone sample (Table 1). Standard measurements (length, height, width) of a subsample of 38 specimens (range 536.58–4390 μm shell length) showed that shell length is strongly correlated with both width and height (R^2 = 0.98 and 0.99, respectively); therefore, only length was used as a measure of size throughout this study (4895 individuals). Regression analysis of shell dimensions, length-frequency histograms and statistical tests were performed with the GraphPad Prism v.6.0 (GraphPad Software, La Jolla California USA, www.graphpad.com). Length-frequency distributions were plotted using a 200-μm length-class interval, chosen according to three criteria (Kelly & Metaxas 2008): (i) most size classes must have at least five individuals, (ii) the number of adjacent empty classes must be minimized, and (iii) the length-class interval should be larger than the errors of the length measurements (12.5, 31.24 and 50 μm, respectively at 40×, 16× and 10× amplifications). Length-frequency distributions were compared with a normal distribution using a Kolmogorov−Smirnov one-sample test. Within a deployment period, length-frequency distributions were compared between pairs of bone samples using Kolmogorov−Smirnov two-sample tests, and the same test was used to compare distributions between the two sampling phases (pooled samples from 18- and 28-month deployments). Modal decomposition of length-frequency distributions was carried out using the MIXDIST package v.0.5–4 (MacDonald & Du 2012) developed for the R environment (v.3.1.1) (R Core team 2014), assuming that mytilid sizes follow a gamma distribution within a single cohort. MIXDIST uses maximum likelihood criteria to provide the best mathematical fit between theoretical and observed mixtures of frequency distributions.

Results

Molecular identification

Our genetic analyses of 29 mussels generated 16 novel mtCOI sequences of 560 bp length with a maximum K2P distance of 1.2% among sequences. The COI sequences exhibited 17 polymorphic sites, of which 13 were singletons and four parsimony informative. The K2P distance among our Setubal specimens was higher than the one reported among specimens from the Marmara Sea (0.6%). The K2P distance between these two operational taxonomic units was 1.2%, and the maximum between the Setubal sequences and the one from the North Atlantic was 0.8%. The median joining network (Fig. 2B) built to depict haplotype relationships revealed that the most common haplotype occupies a central position in the network and is shared by 12 specimens from Setubal, one individual from the North Atlantic identified as '*Idas*' *simpsoni* (Marshall, 1900) and three individuals from the Marmara Sea not previously assigned to any known bathymodiolin species (Ritt *et al.* 2012).

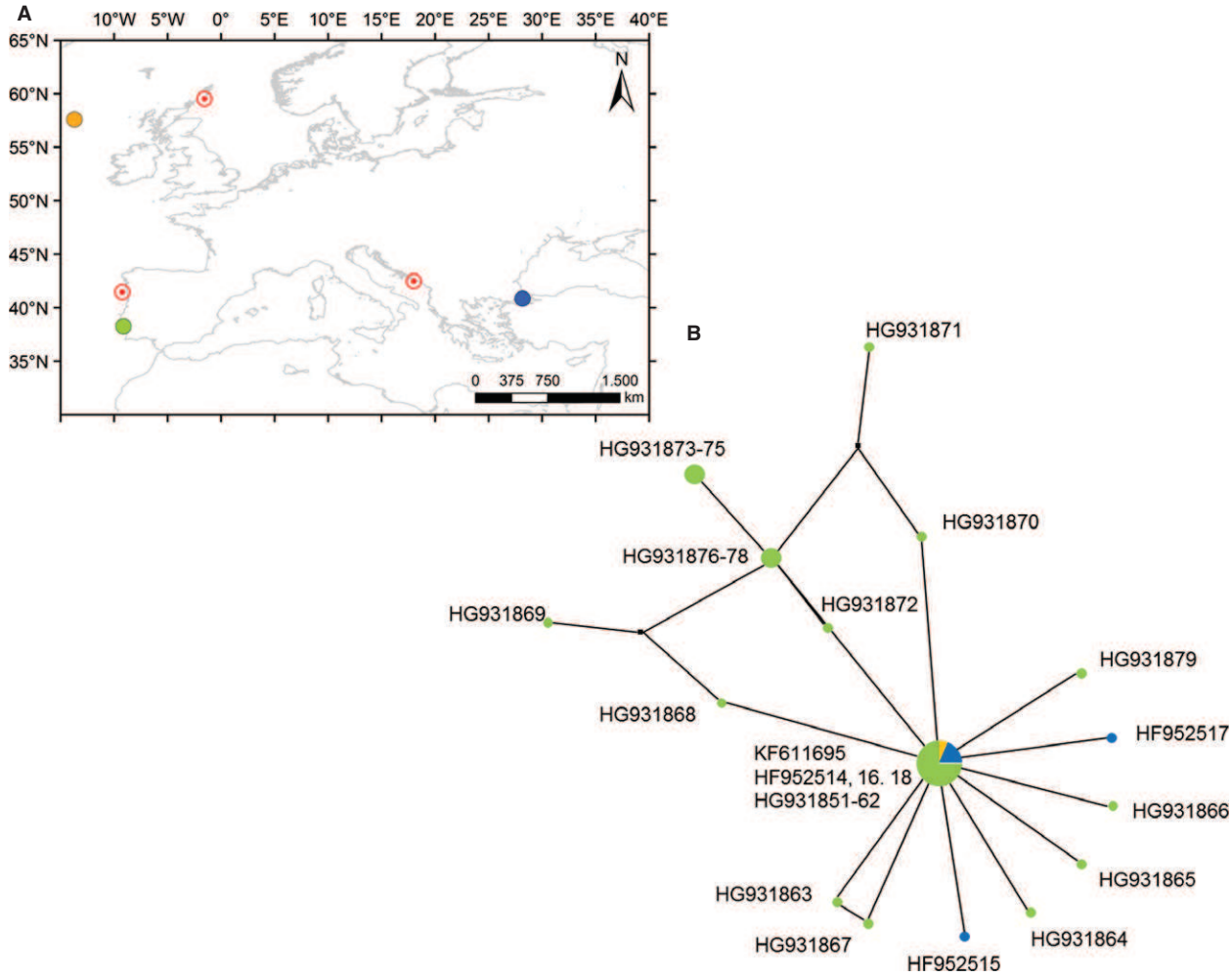

Fig. 2. Taxonomic identification of mussel specimens recovered from cow bones deployed in Setubal Canyon, NE Atlantic. (A): Geographic location of current records of '*Idas*' *simpsoni*. (B): Median-joining network showing the relationships among haplotypes. Each circle represents a different haplotype, and its size is proportional to its relative frequency. Haplotype colors indicate their geographic location as in (A): green, this study (HG931851–879); blue, Marmara Sea (HF95214–18) and orange, off Rockall in the Northern Atlantic (KF611695). Open dotted circles in red indicate records of '*I.*' *simpsoni* without molecular information (see references in text).

Two distinct bacterial phylotypes were retrieved from the 80 partial 16S rRNA gene sequences. The dominant phylotype, to which 79 of the 80 clones belonged, differed by only two out of 1500 bp (99.9% of nucleotide positions identical) from the phylotype of the sulphur-oxidizing symbiont found associated with mussels from the Marmara Sea (JQ038225). The second phylotype occurred only once, belonging to the Bacteroidetes (CFB group), and presented a 92% similarity with the Bacteroidetes phylotype found in *Idas modiolaeformis* collected from the Mediterranean Sea (AM402958). The APS reductase-encoding gene codes for an enzyme responsible for a key step in the sulfur oxidation pathway. The amino acid sequence obtained for the dominant phylotype showed 98% similarity with that from a mussel recovered on a coconut leaf (BC288) in the Bohol Sea (Western Pacific, AM851095).

Size structure

Length-frequency distributions were generated from measurements of 4895 mussels collected from the surfaces of four cow bones deployed in Setubal Canyon (Table 1). Shell lengths ranged from 425 to 6375 μm for the bones collected after 18 months of carcass deployment, and 375–7000 μm for the 28-month bone samples (Table 2). The largest individual (13.8 mm) was found on a separate bone from the 18-month deployment and

Table 2. Summary of shell length range, modal decomposition of length distributions, number of plantigrades and mean prodissoconch (PD) II length of '*Idas*' *simpsoni* collected from cow bones deployed in Setubal Canyon, NE Atlantic. Modes (μ), standard deviations (σ) and proportions (π) of modal components were estimated with the MIXDIST package.

Samples	Shell length range (μm)	Modal components		Number of plantigrades	Mean PD length (μm)
		M1 (μm)	M2 (μm)		
Bone B	450–2550			8	478.91
μ		904.87	–		
σ		331.13	–		
Bone C	425–6375			12	468.75
μ		1099.63	–		
σ		454.71	–		
Total 18-month	425–6375			20	472.81
μ		1031.14	–		
σ		425.68	–		
Bone G	400–4900	χ^2 = 29.07; df = 26; P = 0.31		46	460.19
μ		480.40	1691.80		
σ		102.00	722.70		
π		0.18	0.82		
Bone H	375–7000	χ^2 = 30.00; df = 38; P = 0.15		267	457.54
μ		576.60	800.20		
σ		17.64	965.47		
π		0.46	0.54		
Total 28-month	375–7000	χ^2 = 36.72; df = 27; P = 0.10		313	457.93
μ		459.00	1668.00		
σ		103.10	779.50		
π		0.53	0.47		

was not included in these analyses (Fig. 3). All distributions were significantly different from the normal distribution (Kolmogorov–Smirnov one-sample test, $P < 0.0001$). For the 18-month deployment period, length-frequency distributions of the two bone samples were significantly different (Kolmogorov–Smirnov two-sample test, $P < 0.0001$), but both displayed a unimodal structure characterized by a large number of individuals of approximately 1 mm in length (Fig. 4). A total of 20 plantigrades (newly settled individuals, Fig. 3) was found on bones B and C, representing less than 1% of the mussel population collected after 18 months of carcass deployment. The number of individuals found on bone C was nearly twice the number of mussels on bone B, accounting for highly discrepant densities in these two samples (1.92 and 6.27 individuals·cm^{-2}). By contrast, the length distributions of samples collected after 28 months showed a bimodal structure with clearly distinct modal peaks (Table 2, Fig. 4) and were also significantly different (Kolmogorov–Smirnov two-sample test, $P < 0.0001$). The two bones collected in the second recovery period revealed similar mussel densities (2.05 and 2.58 individuals·cm^{-2}) with an evident size segregation (Tables 1 and 2). A very high percentage (82%) of mussels on bone G were large specimens of about 1.6 mm in length, while on bone H nearly 50% of the mussels were small, with lengths under 600 μm, and including 267 newly settled individuals.

Growth rate

Settlement size was estimated by measuring the length of prodissoconch II (*i.e.* the larval shell) on recently settled specimens (Fig. 4). The mean length of prodissoconch II, obtained from a pooled sample of 666 plantigrades collected over the two sampling periods, was 459 μm (SD 22 μm, range 400–563 μm). Assuming the highest estimated consumption rate of cetacean carcasses in the Atlantic (0.4 kg·h^{-1}), the early phase in which mobile scavengers remove the soft tissues, thereby exposing the bones to bone specialists, may have taken approximately 2 months, which would result in a bone colonization period of 16 months until the first sample recovery after deployment (Jones *et al.* 1998). It is also possible that the bones were scavenged very early on and so were exposed for the entire 18-month period. If we assume that the largest specimen recovered in August 2012 (13.8 mm shell length) was among the first mussel colonizers, the shell growth rate of settled mussels would be 766–869 μm·30 days^{-1}. A comparison of previously estimated growth rates among bathymodiolin mussels in chemosynthetic habitats is shown in Table 3.

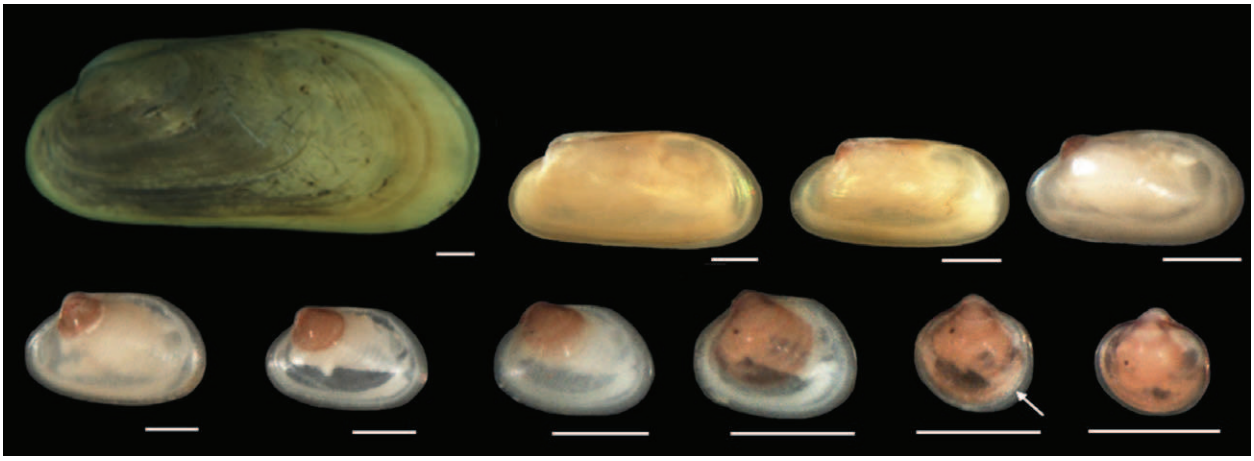

Fig. 3. Digital photographs of '*Idas*' *simpsoni* collected from the surface of cow bones deployed in Setubal Canyon, NE Atlantic. White arrow indicates boundary between prodissoconch II and dissoconch in settled individuals. Plantigrade stage (with no visible dissoconch growth) is shown in the bottom right-hand corner. Scale bars: upper row = 1 mm; lower row = 500 μm.

Discussion

Identification and biogeography of mussels and their symbionts

Shell morphologic traits are poor characters for mytilid species identification as their shells are almost featureless, show great variability of size and shape within molecular units, and convergence of shell shape is observed between distantly related lineages (Lorion *et al.* 2010). The small size of most species found at organic falls has discouraged descriptions of their anatomy, and molecular approaches became routinely used for identification of ESUs. However, a large number of organic-fall species previously recognized in the genus *Idas* have yet not been sequenced. From the 13 nominal species assigned to this genus in the Atlantic and the Mediterranean Sea (Gustafson *et al.* 1998; Pelorce & Poutiers 2009; Giusti *et al.* 2012), only three have DNA sequences available for comparison, namely *Idas modiolaeformis*, *Idas macdonaldi* and '*Idas*' *simpsoni*. Molecular analysis showed that mussels from our samples are genetically identical (K2P distance 1.2%) and 12 out of 29 COI sequences could not be discriminated from the '*I.*' *simpsoni* specimen found on whale bones off Rockall (North Atlantic) or from another three specimens collected on carbonate crusts associated with cold seeps in the Marmara Sea (Ritt *et al.* 2012; Thubaut *et al.* 2013a). The maximum distance estimated among the Setubal, Marmara and Rockall sequences (0.8−1.2%) is similar to previously reported levels of intra-specific variation among bathymodiolin mussels (Lorion *et al.* 2012). Therefore, we conclude that both the Setubal and Marmara specimens are conspecific with '*I.*' *simpsoni*. '*Idas*' *simpsoni* is included in a group of small wood and bone mussels that has been provisionally assigned a new genus name *Nypamodiolus* (Thubaut *et al.* 2013a). As the revised genus-level classification of bathymodiolin mussels has not yet been formally accepted, we continue to use the name '*Idas*' in inverted commas to refer to the species found in this study. The Setubal Canyon and Marmara Sea populations of '*I.*' *simpsoni* are found at considerably greater depths (1000−1120 m) than previously reported for this species (100−430 m, Pelorce & Poutiers 2009; and 500−700 m, Giusti *et al.* 2012) and its known geographic distribution from southern Iceland and the North Sea to the Mediterranean is now further extended to the Marmara Sea.

Although most bathymodiolin species are exclusively found in one type of reducing habitat (vent, seep, wood or bone), a few species are known that colonize multiple types of chemosynthesis-based environments (Sasaki *et al.* 2005; Southward 2008; Lorion *et al.* 2012). '*Idas*' *simpsoni* has been reported from old whale or dolphin skeletons or drifting pieces of blubber, occasionally on wood, and also in rock chippings with adhering drill mud and carbonate crusts associated with seeps (Warén & Carrozza 1990; Southward 2008; Ritt *et al.* 2012). The presence of '*I.*' *simpsoni* on other substrates than those previously reported, here cow bones, highlights the importance that ephemeral substrates in the deep sea may have in the biogeography of chemosymbiotic species. The mussel abundances found in this study indicate that cow bone is a suitable substrate for '*I.*' *simpsoni*, providing a hard substratum for settlement and enough sulfide for the maintenance of the bacterial symbionts. The densities observed in individual bone samples (1.92–6.27 individuals·cm^{-2}) exceed the maximum density (0.46 individuals·cm^{-2}) reported for the '*I.*' *simpsoni* population found on drill

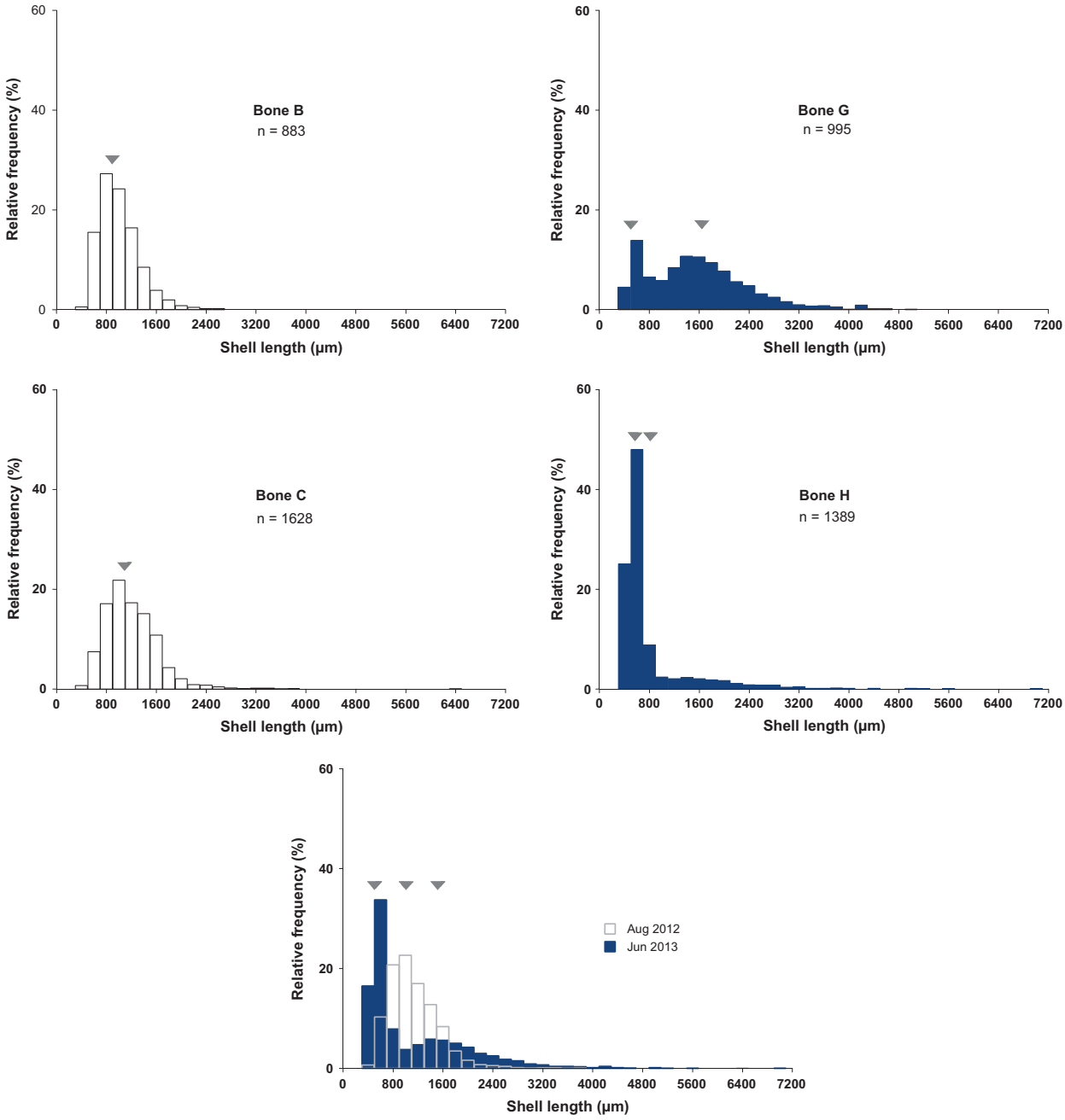

Fig. 4. Length-frequency distribution of '*Idas*' *simpsoni* collected from the surface of cow bones placed in Setubal Canyon, 18 and 28 months after deployment (respectively, bones B and C in August 2012, and bones G and H in June 2013). Grey arrows indicate the mean length of modal components identified with the MIXDIST package; n is the total number of individuals in the distribution.

cuttings in the North Sea (Southward 2008), and are also considerably higher than the *Idas washingtonius* densities reported for Pacific whale carcasses (0.046−0.188 individuals·cm^{-2}, Bennet *et al.* 1994). Although cow carcasses are atypical oceanic food falls, the occurrence of terrestrial vertebrate bones in the deep sea may in fact be more common than previously expected as a result of galley waste and dumping of dead livestock during transport (Vrijenhoek *et al.* 2008; Ramirez-Llodra *et al.* 2011). Nevertheless, the successful colonization of bone falls may be determined by a combination of factors, including the lipid content and longevity. In contrast to the previous experiment of Kitazato & Shirayama (1996), the large amount (>500 kg) of carcass material deployed in Setubal

Table 3. List of Bathymodiolinae species for which shell growth rate has been estimated.

Species	Habitat	Estimated growth rate (μm 30 day^{-1})	References
'*Idas*' *simpsoni*	Cow bone	766–869	This study
Idas modiolaeformis	Wood, carbonate crusts	13–523	Gaudron *et al.* (2012)
Idas argenteus	Wood	39	Dean (1993)
Bathymodiolus azoricus	Vent	904	Nedoncelle *et al.* (2013)
'*Bathymodiolus*' *childressi*	Seep	1440	Arellano & Young (2010)
Bathymodiolus thermophilus	Vent	2100	Rhoads *et al.* (1981)

Canyon was sufficient to support a highly diverse macrofaunal assemblage with numerous representative taxa of the 'opportunist/enrichment', 'sulphophilic' and 'reef' stages that typically characterize organic-fall ecosystems (Hilário *et al.* this issue).

Population structure and settlement patterns

The size-frequency distributions and estimates of shell growth rates of the mussel populations found on the cow bones suggest that settlement of '*Idas*' *simpsoni* is continuous and that post-settlement mortality affects the population structure. While the samples collected after 18 months of carcass deployment showed unimodal length-frequency distributions, which are typical of populations with continuous settlement (Kelly & Metaxas 2008), the 28-month frequency distributions revealed distinct peaks, indicating a discontinuous settlement pattern. However, at each sampling time, the maximum attained shell length was similar (~7 mm) and bone samples showed dominance of mussels of distinct size classes, suggesting spatial segregation of the sizes. Newly settled individuals (plantigrade stage) were found at both sampling times, but they occurred in much larger abundances on the 28-month samples (13% of total individuals in bones G and H, compared to <1% in bones B and C). Using the estimated shell growth rate (766–869 μm·30 days^{-1}), and back-calculating the settlement time of the largest individuals in the population, we concluded that settlement events occurred in March 2011, December 2011–January 2012, August–October 2012 and June 2013; thus, no seasonal pattern in settlement could be detected.

Seasonal reproduction has been reported in large mussels inhabiting hydrothermal vents and cold seeps (Dixon *et al.* 2006; Tyler *et al.* 2007). Other studies on reproduction of small organic-fall mussels could not confirm seasonality due to a lack of samples over different seasons (Tyler *et al.* 2009; Gaudron *et al.* 2012), but continuous recruitment of '*I.*' *simpsoni* was tentatively suggested in the Marmara Sea carbonates (Ritt *et al.* 2012). Regardless of spawning periodicity, viable larval pools may be kept over periods of time that extend all year round owing to the long pelagic larval duration estimated for bathymodiolin species (1–5 months in *Idas modiolaeformis* and up to 13 months in '*Bathymodiolus*' *childressi*), thus allowing continuous settlement as suitable habitat becomes available. Intra-specific competition processes, especially adult–larval interactions such as competition for space and food resources, including larviphagy, play key roles in determining the habitat available for successful larval settlement (Comtet & Desbruyeres 1998). As large individuals become less numerous, the amount of suitable habitat increases, leading to the establishment of another generation of a numerically dominant size-class (Dean 1993). The small number of individuals in our samples with a shell length higher than 3 mm indicates that growth and survival were limited, as '*I.*' *simpsoni* is known to attain considerably higher shell lengths (up to 45 mm, Warén & Carrozza 1990), hence explaining the observed differences in size-class abundances on each bone sample.

Mortality of large-sized specimens may have been caused by insufficient energy and limited space provided by the cow bones. For instance, previous reproductive biology studies on organic-fall mussels have revealed protandric hermaphroditism, with mature males being found with shell sizes of 1.7–7 mm and most well-developed females being larger than 6–8 mm in length (Dean 1993; Tyler *et al.* 2009; Gaudron *et al.* 2012; Laming *et al.* 2014). Thus, it is possible that '*I.*' *simpsoni* individuals were unable to survive the transition stage in the present study, as females normally require larger amounts of energy than males for gamete production (Young 2003). Developing sperm cells were recognized in individuals as small as 1.8 mm, and the largest individual found in the whole experiment (13.8 mm shell length) showed female reproductive features (A. Hilário, personal observation). Similar to the low (10%) relative proportion of females usually observed in the former studies, it is very likely that a reduced number of females could have survived in the cow bones deployed in Setubal canyon.

'*Idas*' *simpsoni* exhibits the fastest shell growth rate among known organic-fall mytilids, but a comparable higher rate is found among seep and vent mussels (Table 3). However, differential growth among individuals of the same population and among different populations cannot be disregarded because it is still unclear how the

interaction of environmental factors (e.g. oxygen levels) and access to energy sources determines bathymodiolin growth (Arellano & Young 2010). The feeding habits of the three organic-fall taxa studied here are quite distinct, which may have contributed to the different estimated growth rates. '*Idas*' *simpsoni* from the present study is associated with a bacterial phylotype that is strictly identical to that associated with specimens from the Marmara Sea. The occurrence of an APS reductase-encoding gene indicates that the dominant symbiont is a sulfur-oxidizing bacterium, as previously documented in various *Idas*-like mussels, including specimens from the Marmara Sea (Ritt *et al.* 2012). By contrast, *I. modiolaeformis* can harbor up to six phylotypes of symbiotic bacteria, depending upon the location from where specimens are sampled (Duperron *et al.* 2008b; Rodrigues *et al.* 2013) and *Idas argenteus* is known to obtain its nutrition only from suspension feeding, ingesting the larvae of a common wood-boring bivalve of the genus *Xyloredo* (Ockelmann & Dinesen 2011).

Conclusions

Bone samples retrieved from the experimental deployment of cow carcasses in Setubal Canyon (NE Atlantic) over more than 2 years revealed continuous settlement of '*Idas*' *simpsoni*, a chemosymbiotic mussel known mainly from cetacean bones but also found on carbonates near seeps. Post-settlement density-dependent mortality of male adults with the likely survival of a small number of females affected the population structure, but effective recruitment may still have been possible, contributing to the dispersal of the species. '*Idas*' *simpsoni* has an opportunistic strategy, with shell growth rates revealed in this study being the fastest among three mussel species occurring at organic substrates in the Atlantic Ocean. This study has provided evidence of the role of organic falls in facilitating dispersal between reducing habitats and highlights the importance of these substrates with respect to the biogeographic distribution of chemosymbiotic species.

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